CYTOTOXIC EFFECTS OF A NOVEL NITRIC OXIDE DONOR COMPOUND AND ONCOGENIC TRANSFORMATION OF A HUMAN UROTHELIAL CELL LINE

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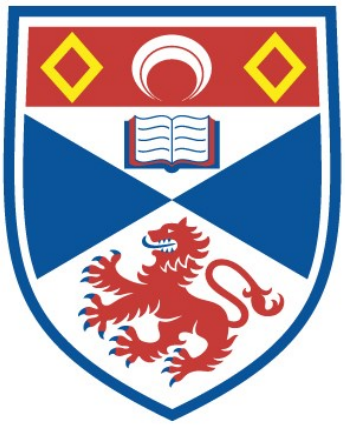

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Cytotoxic effects of a novel nitric oxide donor compound and oncogenic transformation of a human urothelial cell line

Thesis submitted for the degree of Doctor of Philosophy to the

University of St. Andrews

by

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Declaration

I, Hsiao-Hsien Wang, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Signed Hsiao-Hsien Wang Date 09/11/94

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No 12 on 1st October, 1991 and as a candidate for the degree of Ph.D. on 1st October, 1992.

Signed Hsiao-Hsien Wang Date 09/11/94

Certificate

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the Degree of Ph.D.

Signature of Supervisor Date 9/11/94

Andrew C. Riches PhD

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Abstract

Transitional cell carcinoma of the bladder is commonly encountered in urological practice. It affects people of a relatively young age causing economic and social distress to patients. In order to prevent the disease it is important to understand its pathogenesis.

In this study, we have tumorigenically transformed a human urothelial cell by growing cells in a serum free, factor free, chemically defined culture. The tumorigenic property of the cell was determined by the generation of a tumor after inoculation into nude mouse.

DNA fingerprinting analysis demonstrated the common back ground of the non-tumorigenic human urothelial cell and its tumorigenic transformant. This result also shows evidence of mutation occurring during transformation.

By analysing conditioned medium, a significant reduction in the levels of soluble human stem cell factor and interleukin 1α were found in tumorigenic cell conditioned medium.

A model derived from this evidence may suggest that tumor cells undergo further transformation under nutrient and growth factor deprived conditions.

Intravesical chemotherapeutic agents in current use have shown moderate tumor-killing effects with some systemic or local side effects. Identification of a drug with better effect and less side effects is essential for the successful treatment of bladder tumors.

Nitric oxide (NO) is a natural product of the human body with a role in tumor cell-killing. Thus by using NO as a chemotherapeutic agent we could at least expect limited side effects.

Roussin's black salt (RBS) is a novel NO donor. Its cytotoxicity was tested on tumorigenic (T24) and non-tumorigenic (SV-HUC-1) human urothelial cells. The cytotoxicity of RBS was shown to be dose- and contact time- dependent. This cytotoxicity was enhanced by light irradiation and reduced in the presence of haemoglobin.

The cytotoxic effect of RBS was also tested on CHO cells and the DNA repair deficient mutant xrs-5 cell line. Both colony forming and micronuclei forming assays demonstrated that xrs-5 cells are more sensitive to RBS than their counterpart. This result may indicate that NO is involved in the cytotoxicity of RBS and furthermore that DNA damage might be one possible mechanism by which the cytotoxic effect of RBS is expressed.

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Chapter I

Introduction

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Purpose of present studies

1-1 Cancer is a disorder of cells

It is not possible to define a cancer cell in absolute terms. Cancers are fully developed tumors with malignant nature which present with the ability to invade and destroy the surrounding tissues and to spread to other parts of the body where they may generate secondary tumours. The normal division and differentiation of somatic cells in multicellular organisms requires a precise control mechanism in the organism. Tumor cells differ from normal cells in that they demonstrate the failure of such controlling mechanisms, and have achieved a degree of autonomy.

1-1-1 Clonal origins of cancer

Evidence from (1) studies of women heterozygous for the 2 allelic forms of the enzyme glucose-6-phosphate dehydrogenase, the gene for which is on the X-chromosome, demonstrated that the same member of the X-chromosome pair is functional in all cells of a given tumor (Linder, D. *et al* 1965), (2) immunological studies have shown that all cells in a B-cell lineage tumor synthesize the same immunoglobulin (Martensson, L. 1963), (3) cytogenetic studies have shown that all the cells of a tumor present a specific abnormal karyotype (Nowell P. C. 1975) which indicates that most cancers may arise as a clone from a single cell of origin. However, subpopulations which differ in sensitivity to cytotoxic drugs and in genetic stability have been isolated from a fresh primary tumor (Shapiro, J. R. *et al* 1981). Indeed, the cells in most tumors are quite heterogenous due to their adaptation for survival. They survive natural and artificial selection through heterogeneity by producing

new variants and by utilizing subpopulation interactions to counteract any destructive influence (Heppner, G. H. 1984). A similar concept is hypothesized as "clonal evolution" which encompasses two major principles (1) tumors are clonal in origin (2) tumors progress on the basis of genetic instability within the neoplastic population leading to the sequential emergence of mutant subpopulations with increasingly malignant properties (Nowell, P. C. 1976).

But in view of the large numbers of cells which are at risk in an adult organism, the relative rarity of cancer and analysis of the characteristics of clones of experimental tumors indicate that tumors have a multicellular origin (Reddy, A. L. *et al* 1979). A proposal that several transformed cells come together to create a microenvironment which permits their unlimited proliferation and production of a malignant lesion has been made, and that it is the cumulative effect of selective pressures which leads to a monoclonal state (Woodruff, M. F. A. *et al* 1982; Alexander, P. 1985). Regardless of whether neoplasms are multiclonal or monoclonal in origin, tumor cells progress to heterogenous subpopulations with a wide range of phenotypic characteristics by the time they can be clinically diagnosed (Poste, G. *et al* 1980).

1-1-2 Stem cell model of tumor growth

In normal epithelial tissue growth, cell production and cell loss must be balanced to maintain steady state and the total cell population can be regarded as constituting a cell division hierarchy with stem cells at one extreme and differentiated cells at the other extreme. Stem cells have two critical functions that is clonal

expansion and self renewal. In clonal expansion stem cells may act as the initiating cell in a cell division and differentiation process eventually giving rise to the entire clonal hierarchy. With respect to cell renewal, stem cells may undergo cell division to produce stem cell daughters which replace the stem cell used in clonal expansion. Differentiated cells are considered to have little or no capacity for proliferation (Cairns, J. 1975). Since human tumors arise predominantly in tissues which function as stem cell systems in the normal situation and tumors are tissue specific, a stem cell model of tumor growth has been postulated (MacKillop, W. J. *et al* 1983) and this concept predicts that in addition to stem cell and differentiated end cells, another category of proliferating, non-renewing transitional cell exists within the total tumor cell population. Several pieces of evidence such as tissue-specific cell differentiation existing in tumors and only a fraction of cells from human tumors exhibiting self renewal capacity, support the view that stem cells exist in tumor masses. In the case of tumors arising in conditionally renewing tissues such as liver the applicability of this model is doubtful (Buick, R.N. *et al* 1984). Although a small fraction of tumor cells may form colonies in a semisolid medium and the ability of a cell to form a colony implies substantial proliferating capacity (Hamburger, A. W. *et al* 1977), this does not necessarily equate with a stem cell. Stem cells are responsible in vivo for manifestations of tumor growth while clonogenic cells demonstrate a proliferating subpopulation in tissue culture (Steel, G. G. 1977; Buick R. N. *et al* 1984).

1-1-3 Metastasis

Metastasis is the development of secondary tumors that are not in direct contact with the initial primary neoplasm. The ability to produce metastasis is a property of malignant tumor cells although this phenomenon can be observed in non-cancer conditions eg. fragments of chorionic tissue spread to lung or liver during developing of placenta (Douglas, G. W. *et al* 1959) but cancer cells will keep on proliferating in that remote area while chorionic tissue will die and disappear with the cessation of pregnancy. Using modern surgical techniques and aggressive adjuvant therapies, most local primary cancer can be eradicated and the majority of cancer deaths are caused by metastases.

To successfully metastasize, cancer cells require several distinct properties that enable them to invade locally and penetrate into blood vessels or lymphatics, evade of host immune defence mechanisms, arrest and implant in the vessel wall at new locations, undergo extravasation into surrounding tissue and establih new growth (Poste, G. *et al* 1980). Various factors have been proposed as being important in the malignancy of cancer cells, including proteolytic enzymes, tumor angiogenic factors, platelet agglutinating capacity, adhesion molecules, extracellular matrix components such as laminin and fibronectin and major histocompatibility complex gene products (Collard, J. G. *et al* 1988). Clinical observations have shown that certain types of tumors tend to metastasize to specific target organs and thus a "soil and seed" theory has been proposed (Paget, S. 1889) i.e. that cancer cells survive in a "favorable" place and cause a secondary tumor there. Another explanation is the haemodynamic model

which relates the site of metastasis to the number of cancer cells delivered to the organ or the first capillary bed they encounter in the blood flow (Sugarbaker, E. V. 1981) But in an experiment in which tumor cells were intracardiacally injected into animals, although the number of tumor cells distributing to each target organ was correlated to the blood volume they received, the incidence of metastsis of each organ did not show the same correlation (Murphy, P. *et al* 1985). So it seems that both hemodynamic factors and selective growth of certain cancer cells in certain organs contribute to the influence of the organ specific metastasis of human cancers.

1-1-4 Cancer associated with DNA alterations

Cancers appear to arise as a result of multiple changes in the cellular genome leading to distortion of either the expression or biochemical function of genes. There is much evidence to support the contribution of non-random chromosomal alterations, in the form of structural or numerical aberrations, to human cancer development and progression (Sandberg, A. A. *et al* 1988; Yunis, J. J. 1983; Rowley, J. D. 1984). Several congenital diseases associated with increased chromosome breakage, for example Down's syndrome and Klinefelter's syndrome, have an increased incidence of cancer (Gardener-Medwin, D. 1987; London, D. R. 1987). Individuals with genetically determined traits associated with a deficiency in the enzymes necessary to repair lesions in DNA, such as Bloom's syndrome (BS) and Xeroderma pigmentosum (XP), are strongly predisposed to develop malignant diseases (Gordon-Smith, E. C. 1987). In addition Wilm's tumor and retinoblastoma are two

examples of many heritable human cancers (Chantler, C. 1987; Harris, H. 1987). Finally almost all agents associated with neoplastic transformation (carcinogen) are known to cause DNA mutations (Ames, B. N. 1979). All these examples support the evidence for the genetic origin of cancer and have been linked by the discovery of oncogenes (Bishop, J. M. 1987).

1-1-5 Oncogenes

The term "oncogene" was originally used to denote a hypothetical class of genes which is a covert viral gene that become activated in a host cell to cause tumor formation (Huebner, R. J. *et al* 1969). This concept, namely that there are genes capable of causing cancer, is based largely on studies carried out with transplantable tumors in chickens, mice and rats. Some retroviruses induce neoplastic transformation in normal cells in culture and susceptible animals. This occurs when certain genes -"viral oncogenes"(*v-onc*) are integrated into the host cell genome. By using molecular hybridization, it has been shown that sequences homologous to the oncogene region of the virus were present in the DNA of normal cells. These cellular homologues of viral oncogenes have been referred to as "cellular proto-oncogenes"(*c-onc*). These proto-oncogenes probably existed in the cellular genome first (Minden, M. D. *et al* 1992) and have implanted into viral genome during the recombination between retroviral and cellular genomes. In this new setting the cellular genes may become oncogenic. The genesis of retroviral oncogenes from cellular proto-oncogenes has been called "transduction" (Bishop, J. M. 1987).

Human tumor DNA can be transferred to NIH/3T3 embryo fibroblasts in which it may generate transformed cells. They can proliferate in semi-solid media, do not stop growing when they become confluent (loss of contact inhibition) and may form tumors in animals. Analysis of the genes responsible for the transformation reveals a diverse assortment of oncogenes and most of them are homologous to the viral oncogene H-ras and K-ras (Parada, L. F. *et al* 1982; Bishop, J. M. 1985). Oncogenes identified by gene transfer are mutants of normal cellular genes and the mutations activate the oncogenes (Bishop, J. M. 1987).

Retroviruses, that do not have oncogenes however may also cause cancers. After the integration of viral DNA, the expression of cellular genes is altered by bring them under the control of the viral genome (Payne, G. S. *et al* 1982; Nusse, R. 1986). Such events are called "insertional mutagenesis". Isolation of genes near sites of integration of retrovirus will identify genes responsible for the transformation (Nusse, R. *et al* 1982).

Cancer cells have provided clues to oncogenes in the form of microscopically visible damage to chromosomes. A growing number of genes involved in tumor development have been identified by their association with chromosome translocations (Tsujimoto, Y. 1985) and abnormal amplification of large domains within chromsomes (Schwab, M. *et al* 1983; Kohl, N. E. *et al* 1983).

Proto-oncogenes encode protein whose normal function is to regulate cellular responses to external signals that elicit cell growth and differentiation. These protein products are relays in the elaborate biochemical circuitry that governs the phenotype of vertebrate cells. Although the number of proto-oncogenes is large, their functions can be categorized into three main biochemical

mechanisms: (1) phosphorylation of proteins with serine, threonine and tyrosine as substrates, (2) transmission of signals by GTPases and (3) controlling of transcription from DNA (Bishop, J. M. 1991). It does not appear to be silent "cancer genes" which become activated, rather it seems that normally active genes which serve important cell functions become functinally altered and lead cells separate from their normal milieu, proliferate independently and thus become cancers (Weiner, T. *et al* 1994). Two major lesions in the proto-oncogenes responsible for the tumorigenesis are (1) mutations in the DNA that alter the constitutive activity of gene products and(2) deregulation which results in overexpression of the normal cellular product (Land, H. *et al* 1983b).

1-1-6 Tumor suppressor genes

Tumor suppressor genes are wild-type alleles of genes that play regulatory roles in cell proliferation, differentiation and other cellular and systemic processes. It is their loss, or inactivation, that is oncogenic (Sager, R. 1989). Although several pieces of evidence supporting the existence of tumor suppressor genes are indirect, they are non the less very persuasive. In the case of retinoblastoma it has been found that the loss, or inactivation, of both alleles of the gene (Rb) located near 13q14 which is necessary for proper differentiation of retinoblasts appears to be the primary mechanism in the development of retinoblastoma (Murphree, A. L. *et al* 1984). Evidence comes from somatic cell hybridization experiments which showed that fusion of tumor cells with normal cells almost invariably resulted in the outgrowth of non-tumorigenic hybrids (Harris, H. 1988). The repeated observation of loss of

heterozygosity (LOH) of a specific chromosomal marker in cells from a particular tumor type, suggests the presence of a closely mapping tumor suppressor gene, the loss of which is involved in tumor pathogenesis (Hansen, M. F. *et al* 1987). Oncogenes are identified by their positive role in the transformation of cells, while tumor suppressor genes function in a fundamentally different way. They serve as transducers of antiproliferative signals and as part of the response machinery that enables a cell to stop progressing through the cell cycle, to differentiate, to senesce, or to die (Weinberg, R. A. 1991), thus blocking transformation and driving cells toward normality (Sager, R. 1989). Deletion of genes, alteration of gene expression, or inactivation of gene products are the types of damage found in tumor suppressor genes which cause loss of function (Bishop, J. M. 1991).The progression of many tumors to full malignancy requires both active oncogenes and inactive suppressor genes in the tumor cell genome (Weinberg, R. A. 1991).

1-1-7 Multistep oncogenesis

Tumorogenesis in humans and experimental animals is thought to be a multistep process (Nowell, P. C. 1976). This has been suggested in a number of ways (1) Statistical analysis of age-dependent tumor incidences have suggested multiple successive independent steps for the formation of a neoplasm (Klein, G. *et al* 1985). (2) Pathological studies show that tumors progressively acquire new phenotypes by passing through a series of distinct stages such as anaplasia, metaplasia and neoplasia (Land, H. *et al* 1983b). (3) In animal models the process has been categorized into

initiation, promotion and progression (Farber, E. 1984). Molecular and cellular evidence of multistep tumorigenesis is now becoming apparent. Oncogenes of either cellular, or viral origin, when working alone are unable to transform primary embryo cells to a fully tumorigenic phenotype. They can however induce tumorigenecity when working in collaboration (Rassoulzadegan, M. *et al* 1982; Ruley, H. E. 1983; Land, H. *et al* 1983a). It has been suggested that at least two types of change in cell physiology are needed to convert a normal cell to a fully tumorigenic cell, one occurring in the cytoplasm and one in the nucleus (Weinberg, R. A. 1985; 1989). A study on colorectal cancer has indicated that multiple molecular events are necessary to convert colonic mucosa to an invasive carcinoma and that certain of these events correspond to pathological stages of the disease (Fearon, E. R. *et al* 1990). It seems that malignant tumors arise from a protracted sequence of events, each step in the sequence creates an additional phenotypic aberration (Bishop, J. M. 1987).

1-2 Bladder cancer

Nearly one hundred years ago the occurence of bladder cancer was connected with an environmental agent (Rehn, L. 1895). Many studies have shown that increased incidence of bladder cancer is associated with highly industrialized areas, either world wide (Doll, R. *et al* 1970) or locally (Levin, M. L. *et al* 1960) with highest rates found in the United States and Denmark (Skeet, R. G. 1990). It is the fourth most common cause of cancer deaths among American men over the age of 75 years and the male to female ratio of occurrence is approximately 3:1 (Catalona, W. J. 1991). In Britain it

ranks seventh among the cancers in man and eleventh among women and the highest rate appears in Scotland (Davies, J. M. 1982). In England and Wales bladder cancer develops in about 2% of males and 0.5% of females (Skeet, R. G. 1990). One in six thousand adults will be affected by bladder tumors each year in Scotland (Kaye,. S. B. *et al* 1988). In the Republic of China there is an unusually high incidence of bladder cancer in the southern part of Taiwan province where black foot disease is endemic. It may be due to exposure to arsenic which reaches high levels in the wells of that area (Chen, C.-J. *et al* 1986). Bladder cancer is extremely rare in the first two decades of life, and the incidence begins to rise sharply after the fifth decade of life with over 50% of cases occurring after the age of 70 in both sexes (Skeet, R. G. 1990).

1-2-1 Risk factors

There are several factors which have been described in association with increased incidence of bladder cancer:

1-2-1-1 Tobacco

This is the most important known preventable cause of bladder cancer (Morrison, A. S. 1984).The first clinical study showing the association of smoking and bladder cancer was published in 1956 (Lilienfeld, A. M. *et al* 1956). The mechanism of bladder carcinogenesis of cigarette smoking is suggested to be due to urinary excretion of both alpha and beta-naphthylamine which are urothelial carcinogens (Hoffman, D. *et al* 1969). Relative risk is over two for cigarette smokers compared with non-smokers. A significant dose-response relationship has been shown with respect to increasing cigarette consumption with nearly half of bladder

tumors in men and a quarter in women being directly due to cigarette smoking (Claude, J. *et al* 1986). In addition the severity of bladder tumors is significantly higher in smokers (Thompson, I. M. *et al* 1987). Risk of tumor development appears to decrease following smoking cessation, but only slowly (Howe, G. R. *et al* 1980; Vineis, P. *et al* 1984).

1-2-1-2 Coffee drinking

The association between coffee drinking and bladder cancer has been suggested since 1971 (Cole, P. 1971). A study using nearly 3000 patients and 5800 controls has shown a statistically significant relative risk of 1.6 in males and 1.2 in females for coffee drinkers over non-coffee drinkers (Hartge, P. *et al* 1983). However, in Denmark where caffeine consumption exceeds that of United Kingdom by a factor of 9 and where there is the highest incidence of bladder tumor, no association was noted (Jensen, O. M. *et al* 1986). As coffee consumption and cigarette smoking are often intimately linked, it appears that if there is a relationship between coffee drinking and bladder cancer, it is weak.

1-2-1-3 Artificial sweeteners

A clinical association of artificial sweeteners (saccharin) and bladder tumors has been suggested in Canada. The relative risk of males with a history of saccharin usage over controls is 1.6 but no increase risk was noted in women (Howe, G. R. *et al* 1977). A large scale case control study, conducted by National Cancer Institute, has not substantiated any positive relationship. Only those who

reported very frequent use of artificial sweeteners appeared to have a small elevated risk of bladder cancer and the dose response pattern was irregular (Morrison, A. S. *et al* 1983).

1-2-1-4 Occupational exposure

As mentioned before nearly one centre ago, there has been a description of the development of bladder cancer in workers exposed to aniline dye (Rehn,L. 1895). It has been estimated that between 18 and 35% of all bladder tumors in men are due to occupational exposure, but only 1-6% of tumors in women result from similar exposure (Matanoski, G. M. *et al* 1981). Many compounds are now known to cause urothelial cancer in man, they are all aromatic amines and have a similar chemical structure. Established carcinogens include beta-naphthylamine, benzidine, 4-aminodiphenyl and 4-nitordiphenyl (Wallance, D. M. A. 1990). Several industrial processes have high occupational risk of bladder tumors including 1. chemicals and dyes, 2. rubber and tyres, 3. petroleum, 4. leather, 5. printing, 6. plastics, 7. paints, 8. organic chemicals, 9. clerical work, 10. hairdressing. All these jobs involve contact with agents which increase the risk of bladder tumors (Thompson I. M. Jr. *et al* 1990).

1-2-1-5 Cyclophosphamide

Cyclophosphamide is the alkylating agent in widest clinical use and in many anitcancer treatment protocols. The parent compound is inactive and primary activation takes place in the liver. These active metabolites are transported into cells and probably decomposed

there to acrolein and phosphamide mustard (Erlichman, C. 1992). There is a report of 45-fold increased risk of bladder tumors in patients who had previously received cyclophosphamide when compared with a similar untreated population (Fairchild, W. V. *et al* 1979).

1-2-2 Histopathology

The urinary bladder is lined by a specialized epithelium, the urothelium is composed predominantly of transitional epithelium. The most superficial cells, the umbrella cells, have a unique structure and are integral to the barrier action of the epithelium. Capillaries exist in the connective tissue (lamina propria) immediately deep to the basement membrane, which lies underneath the surface epithelium. The bladder wall consists of 3 loosely interwoven layers of smooth muscle and the outer adventitial coat contains arteries, veins and lymphatics (Wheater, P. R. *et al* 1979 ; Cormack , D. H. 1984). Although the epithelial lining of the urinary bladder constitutes a relatively small proportion of the mass of bladder, its tumors comprise more than 95% of malignancy of this organ. Transitional cell carcinoma accounts for 90% of these cases, squamous cell carcinomas for 8% and adenocarcinoma for the remaining 2% (Olssen, C. A. *et al* 1979).
Carcinoma of the transitional epithelium may be the result of a wide spread biological change which affects most, if not the entire urothelium, if one area has undergone malignant change then it can be assumed that the whole urothelium has been exposed to the same urine borne or endogenously produced carcinogen. This field change concept proposes that subsequent tumors arise from

preneoplastic changes or *in situ* carcinoma, progressive growth of these lesions leads to new occurrence. Evidence supporting this concept is derived from results of selected site or random biopsy materials obtained from visible lesions as well as from apparently normal looking mucosa (Melicow, M. M. *et al* 1952; Althausne, A. F. *et al* 1976; Cooper, T. P. 1977; Soloway, M. S. *et al* 1978). Thus bladder tumors usually present in multifocal lesions either synchronously or successively, so that although a tumor(s) may be successfully eradicated at the time of diagnosis new ones emerge either at the same site (recurrence) or at another site within the bladder (Webb, J. N. 1990). Tumor cell implantation has been demonstrated in the bladder of experimental animals (Weldan, T. E. *et al* 1975) and clinical observation of frequent urethral or bladder neck recurrence of tumors, leads to the implication that the implantation of tumor cells at the time of transurethral resection is an etiological factor in new occurrence of bladder tumors (Hinman, F. 1956). These factors make the management of this disease, especially the superficial bladder tumors, particularly difficult.

1-2-3 Superficial bladder tumors

Approximately 70 to 85 per cent of individuals with bladder cancer will have their initial tumor confined to the mucosa or lamina propria (Torti, F. M. *et al* 1984). Although many of these are easy to treat by endoscopic resection, tumor recurrence occurs after complete resection in about 50-70% of patients. Many tumors recur with a higher degree of malignancy than the primary tumor and in 10-15% of patients the tumor will invade into the muscle layers (Green, L. F. *et al* 1973). It is believed that superficial bladder

cancer constitutes an entity distinct from muscle-invasive transitional cell carcinoma (Kaye, K. W. *et al* 1982). This unique characteristic of superficial bladder tumor requires an additional treatment in most patients as a supplement to endoscopic resection and together with repetitive follow up cystoscopy. Intravesical instillation of drug to treat the disease is the most important option for this purpose.

1-2-4 Intravesical chemotherapy

The anatomic position of the urinary bladder makes it readily accessible from outside the body using a cystoscope or a catheter. Local instillation of anticancer chemotherapeutic agents into bladder would allow for a high concentration of drug to act directly on tumor cells and the entire urothelium of bladder for a relatively long period of time thus inhibiting the growth of pre-neoplastic lesions and eradicating viable tumor cells. The troublesome systemic toxicity of the antineoplastic agents would therefore be kept minimal. Although in normal conditions the urothelium functions as a barrier to prevent the absorption of urine but anticancer drugs can be absorbed through the urothelium in diseased bladder and under non-physiological conditions. Five major factors influence the absorption of drugs through the bladder: molecular weight of the drug, pH of the solution, drug concentration, changes of urothelial surface and length of exposure duration of the urothelium to drug (Torti, F. M. *et al* 1984).

Molecular weight: Studies of various sulfonamides indicate that compounds with molecular weight > 200 AMU are not absorbed in

appreciable amounts. Smaller molecules are absorbed by passive diffusion (Jones, H. C. *et al* 1961).

pH of the solution: The nonionized (absorbable) species of a drug will predominate in a solution whose pH exceeds the pKa of the agent and potentially facilitate the absorption of the drug.

Drug concentration: If passive diffusion is a main mechanism of drug absorption then the concentration gradient of the agent between the plasma and the solution contained in the bladder is a decisive factor in the absorptive process.

Changes in the urothelial surface: As malignant change progresses it has been demonstrated that membranes become more permeable (Soloway, M. S. 1977). Hypervascularity of bladder mucosa appears after surgical instrumentation or infection. Bare areas of bladder wall after for example transurethral resection of tumor, which have lost the protection from urothelium. All of these conditions have a high rate of drug absorption (Pavone-Macaluso, M. *et al* 1976).

Time of drug contact with the urothelium: Data suggest that the main effect caused by increasing contact time may result from toxic substances which cause increased exfoliation with denudation of urothelium (Murphy, W. M. *et al* 1981).

Several drugs have been used as intravesical chemotherapeutic agents in the treatment of superficial bladder tumors. Many of them are abandoned due to their toxicity or lack of therapeutic effect. currently one of the most commonly used drug is thiotepa.

1-2-4-1 Thiotepa as a chemotherapeutic agent

Thiotepa is a polyfunctional akylating agent and following the formation of ethylenimine radicals alkylate biological molecules. Its

main cytotoxic effect appears to be the akylation the bases of DNA causing cross-linking of DNA strands (Erlichman,C. 1992). Its first use as intravesical chemotherapeutic agent was in 1961 (Jones, H. C. *et al* 1961). The usual dose is 1mg/ml and about 2-25% of patients receiving this treatment will develop myelosuppression which is the major side effect of thiotepa (England, H. R. *et al* 1981). When used as a definitive therapy for low grade superficial bladder tumors approximately two thirds of patients have shown a complete or partial response (Torti, F. M. *et al* 1984) As a prophylactic agent, it has been demonstrated that the disease-free interval after transurethral resection of tumor can be significantly prolonged (Schulman, C. C. *et al* 1982), and it also decreases the rate of recurrence (Koontz, W. W. *et al* 1981). The optimal cytotoxic agent for intravesical administration has not been conclusively identified in randomized trials (Raghavan, D. *et al* 1990). Thiotepa has been used widely with minimal toxicity and is currently the standard to which other agents are compared (Soloway, M. S. 1988).

Controlled cellular proliferation and differentiation in vivo involves multiple levels of coordination. A key role however is played by soluble factors including the diverse group of secreted molecules known as the polypeptide growth factors. That growth factors might contribute to the processes of transformation in vivo was suggested by experiments on virally transformed cells and work on the isolation of growth factor genes by their ability to transform mouse fibroblast in vitro. Stem cell factor has been suggested as a pleiotropic growth factor with biological activities in three

migratory cell lineages during embryonal development and postnatal life. It may have oncogenic potential.

1-3 Stem cell factor

Mutation at the *Dominant spotting* (*W*) and *Steel* (S^l) loci in mice, which display a similar phenotype characterized by anemia, lack of hair pigment and sterility, affect the development of three unrelated stem cell populations: neural crest-derived melanocytes, germ cells and hematopoietic precursor cells (Russell, E. S. *et al* 1979). The genes affected by these mutations are known to be located on chromosomes 5 and 10 respectively and to control different cellular processes. Numerous studies have suggested that *W* mutation acts within the stem cell populations. Defects due to the Sl mutation can be rescued by normal environments and its effects are therefore mediated through the microenvironment in which stem cells function (Mayer, T. C. 1970 ; Mayer, T. C. *et al* 1968 ; McCulloch, E. A. *et al* 1964 ; 1965). These data may reflect a receptor-ligand relationship between the products of the W and Sl genes (Chabot, B. *et al* 1988).

1-3-1 W locus

The *W* locus encodes the c-*kit* oncogene, whose gene product (c-Kit) is a transmembrane tyrosine kinase receptor that is structurally similar to the receptors for platelet derived growth factor (PDGF) and colony stimulating factor 1 (CSF-1). Severe *W* alleles (W^{42}) are due to mutation in the kinase domain of c-*kit* DNA

and display no detectable c-Kit kinase activity (Tan, J. C. *et al* 1990), while more subtle phenotypes (W^{44}) result from a structural rearrangement in the genome and appear to be related to the reduced amount of c-Kit protein (Geissler, E. N. *et al* 1988).

1-3-2 Sl locus

The ligand for c-*kit* receptor has been identified as the gene product of the *Sl* locus by several laboratories and given a variety of names which reflect its pleiotropic effects. It includes "Stem cell factor" (SCF) (Zsebo, K. M. *et al* 1990 a ; b), "Kit ligand" (KL) (Flanagan, J. G. *et al* 1990; Huang, E. *et al* 1990), "Mast cell growth factor" (MGF) (William, D. E. *et al* 1990; Anderson, D. M. *et al* 1990), and "Steel factor" (SLF) (Witte, O. N. 1990 ; Morrison-Graham, K. *et al* 1993). SCF will be used as the product of *Sl* locus and refer to "*scf*" as its gene for the discussion followed. Homozygous lethal *Sl* mutation contain a large deletion of the *scf* locus while no, or minor, structural alternations in homozygous, viable *Sl* mutation are observed (Copeland, N. G. *et al* 1990). The Sl^d homozygous mutant mice are viable display severely defect phenotype, and only produce the soluble form of SCF (Flanagan, J. G. *et al* 1991).

1-3-3 Structure of SCF

The nucleotide sequence of the cloned SCF cDNA encodes a predicted transmembrane polypeptide with a leader sequence, extracellular domain, membrane spanning region and short cytoplasmic tail (Anderson, D. M. *et al* 1990). SCF in human and

mouse systems is now known to both contain the 220-amino acid protein and the 248-amino acid protein which is efficiently proteolytically cleaved at a site encoded by the primary mRNA transcript within exon 6 and results in a 164-amino acid, biologically active, secreted SCF. The 220-amino acid form of the protein is produced from an alternatively spliced second mRNA transcript, which lacks the exon 6 sequence and is therefore membrane bound (Toksoz, D. *et al* 1992 ; Flanagan, J. G. *et al* 1991). Murine SCF has two distinct proteolytic cleavage sites. The second site, located in exon 7, is utilized only in the absence of the primary site and this appears to be species-specific, since human SCF remains largely membrane-associated at this site (Majumdar, M. K. *et al* 1994). The secreted form of SCF involves both a separate biosynthetic pathway and post-translational proteolytic cleavage to release the extracellular domain.

1-3-4 Biological functions of SCF

Both the soluble and transmembrane form of SCF have growth factor activities (Anderson, D. M. *et al* 1990). The molecular lesion in the Sl^d allele is a deletion of the transmembrane and intracellular domains of SCF. The presence of a defective stem cell phenotype in this mouse implies that production of soluble SCF is not adequate for normal biological function. This indicates that the membrane-bound form of SCF may be important in mediating cell-cell adhesion and interaction and have a critical biological role in the intact organism (Godin, I. *et al* 1991; Dolci, S. *et al* 1991; Flanagan, J. G. *et al* 1991). Murine, or rat, soluble SCF is highly homologous to its human counterpart and is active on human cells, but the human

protein is 800 fold less active on mouse or rat cells (Martin, F. H. *et al* 1990). Membrane bound SCF increases both the overall numbers and colony sizes of primordial germ cells (PGC), while secreted SCF causes a large increase in the initial survival and motilities of PGC, but does not promote long-term survival in culture (Godin, I. *et al* 1991). Although SCF is required for survival it is not sufficient for proliferation of PGCs in culture (Dolci, S. *et al* 1991).

Both forms of SCF promote the proliferation of mast cell lines. In combination with other factors soluble SCF acts in a synergistic manner to induce myeloid and erythroid lineage colony formation, such factors include erythropoietin (EPO), granulocyte-colony stimulation factor (G-CSF), granulocyte-macrophage-colony stimulation factor (GM-CSF), and interleukin 1ß,3,6,7 (IL 1ß,3,6,7). Soluble SCF itself does not trigger differentiation of hematopoietic progenitors; it enhances their proliferative potential, perhaps playing a role in the activation of primitive haematopoietic cells (Anderson, D. M. *et al* 1990; Martin, F. H. *et al* 1990; Nocka, K. *et al* 1990; Zsebo, K. M. *et al* 1990a).

1-3-5 Soluble SCF decrease in tumorigenic cells

In a number of small cell lung cancers, 88% (22/25) have been found to express c-*kit* mRNA. 76% (19/25) of the examined tumors expressed SCF mRNA and amongst these 95% expressed both forms of mRNA (Rygaard, K. *et al* 1993). Other human solid tumor cell lines express the c-Kit receptor, membrane bound and soluble SCF (Turner, A. M. *et al* 1992). Indicates that this receptor/ligand system may have an autocrine function in tumors (Rygaard, K. *et al* 1993 ; Turner, A. M. *et al* 1992). The c-*kit* proto-oncogene is

expressed in a majority (20/25) of studied acute myeloblastic leukemias in contrast to undetectable expression in normal bone marrow and is functional in terms of its supporting proliferation (Ikeda, H. *et al* 1991). The concentration of soluble SCF in patients with myelodysplastic syndrome (MDS) is significantly lower when compared with normal subjects and this may be due to abnormal SCF producing cells in MDS or an increase in c-Kit receptors on the surface of tumor cells (Bowen, D. *et al* 1993).

Chemotherapeutic agents have been widely used as one option of cancer treatment in recent years. Because of toxicity to normal tissue, at present only a limited choice of drugs is available for clinical use.

Nitric oxide is a natural occurring product of the human body which has a cytotoxicity effect on tumor cells. In this respect it might be a good candidate as a chemotherapeutic agent.

1-4 Nitric oxide

Nitric oxide (NO), a gas under atmospheric conditions, has been elected "The molecule of the year 1992" by the American Association for the Advancement of Science. It accounts for the biological properties of endothelium derived relaxing factor (EDRF) (Palmer, R. M. J. *et al* 1987 ; 1988a), represents a completely novel class of neuronal messenger (Snyder, S. H. *et al* 1992) and is the endogenous stimulator of soluble guanylate cyclase. In addition, NO is an effector molecule released by murine macrophages and other

cells after immunological activation. So far, the only clearly established role for this NO is as a cytotoxic molecule against invading microorganisms and tumor cells (Moncada, S. *et al* 1991 ; Henry, Y. *et al* 1993).

1-4-1 NO is the molecule responsible for the cytotoxicity of activated macrophages

It used to be thought that nitrates (NO_3^-) were derived from dietary intake and environmental exposure, but endogenous synthesis of 10 μmol/Kg body weight per day of NO_3^- was observed in humans which received a low NO_3^- diet (Green, L. C. *et al* 1981b). This process was originally believed to be a product of intestinal microbial metabolism but germ free mammalian animals could synthesis it as well (Green, L. C. 1981a). Rats with a fever induced by intraperitoneal injection of Escherichia coli lipopolysaccharide (LPS) endotoxin had a marked increase in their urinary output of NO_3^- and the amount of this increase correlated with the degree of fever induced (Wagner, D. A. *et al* 1983). These results showed that there is a basal level of NO_3^- synthesis and that this synthesis can be enhanced by stimulation of the immune system. However in experiments carried out in mice, neither functional T-lymphocytes nor LPS-responsive B-lymphocytes, were required *in vivo* and activated macrophages alone were competent *in vitro* to perform the LPS-induced nitrite (NO_2^-)/NO_3^- synthesis (Stuehr, D. J. *et al* 1985). Activated murine peritoneal macrophages in culture and macrophage cell lines demonstrate an increased production of NO_2^-/NO_3^- by the stimulation with interferonγ (IFN γ) LPS and tumor necrosis factor (TNF) (Stuehr, D. J. *et al* 1987b ;

Ding, A. H. *et al* 1988) .This reaction was L-arginine dependent and both the NO_2^- and NO_3^- formed were derived from the terminal guanidino atom(s) of L-arginine (Iyengar, R. *et al* 1987). This process produced L-citrulline as a co-product and was responsible for the cytotoxic activities of macrophages (Hibbs, J. B. Jr. *et al* 1987a). N^G-monomethyl-L-arginine (L-NMMA) prevented the synthesis of both of these products as well as the expression of cytotoxicity (Hibbs, J. B. Jr. *et al* 1987b). An enzymatic activity in the activated macrophages converts L-arginine into an intermediate chemical. The chemical turned out to be nitric oxide (NO), which quickly oxidized into NO_2 and NO_3 (Marletta, M. A. *et al* 1988; ; Hibbs, J. B. Jr. *et al* 1988 ; Stuehr, D. J. *et al* 1989). When macrophages are activated by endotoxins, lymphokines, and tumor cells (Nozaki, Y. *et al* 1993) they respond by converting L-argininein to nitric oxide. In addition to macrophages and endothelial cells (Ignarro, L. J. *et al* 1987), neutrophils (McCall, T. B. *et al* 1989), Kupffer cells (Billiar, T. R. *et al* 1989), microglial cells (Merrill, J. E. *et al* 1993), hepatocytes (Curran, R. D. *et al* 1989), adenocarcinoma cell line EMT-6 (Lepoivre, M. *et al* 1989), and cells from cerebellum (Bredt, D. S. 1989), and adrenal gland (Palacios, M. *et al* 1989) can generate these L-arginine-derived molecules.

1-4-2 Cytotoxicity of nitric oxide

Nitric oxide (NO), a short-lived , lipophilic, free radical is the primary nitrogen oxide formed by macrophages and some other cells in response to lyphokines and immuno-stimulants. NO gas causes the same toxic pattern to tumor cells as activated

mcarophages (Hibbs, J. B. Jr. *et al* 1988). The characteristics of toxicity include the inhibition of mitochondrial respiration, the inhibition of DNA synthesis and the inhibition of the citric acid cycle enzyme aconitase, which appear in target cells and also affect the activated macrophages themselves (Drapier, J.-C. *et al* 1988).The mechanism by which NO kills cells is presently unknown, but several possible types of cytotoxicity have been proposed and summarized in the table 1-1.

1-4-2-1 Free radicals

Reactive free radical species are associated with several forms of tissue damage and disease and also with the process of ageing (Fridovich, I. 1983 ; Sies, H. *et al* 1992 ; Rose, R. C. *et al* 1993). NO is a free radical possessing an extra electron making it highly chemically reactive and therefore extremely labile. Superoxide (O_2^-) is a commonly encountered intermediate of oxygen reduction in both biotic and abiotic systems and this free radical constitutes a threat to the chemical integrity of living cells (Fridovich, I. 1983). NO is known to rapidly react with superoxide to form the stable peroxynitrite anion which itself demonstrates a cytotoxic effect against *Trypanosoma Cruzi* (Denicola, A. *et al* 1993). It has been proposed that peroxynitrite decays to nitrogen dioxide and an hydroxyl radical which is considered the strongest oxidant in biological systems (Beckman, J. S. *et al* 1990). Further more, experiments have demonstrated that *in vitro* NO reacts with hydrogen peroxide to release large amounts of chemiluminescence with the characteristics of the highly cytotoxic species, singlet oxygen (Noronha-Dutra, A. A. *et al* 1993).

1-4-2-2 Inhibition of mitochondrial respiration

The failure of respiration is caused by the nearly complete inhibition of the nicotinamide adenine dinucleotide (NADH):ubiquinone oxidoreductase complex, the succinate:ubiquinone oxidoreductase complex (Granger, D. L. *et al* 1982) and aconitase (Kilbourn, R. G. *et al* 1984), all iron-sulphur (Fe-S) associated enzymes (Hibbs, J. B. Jr. *et al* 1990). Exposure of Fe-S groups to NO results in nitrosyl-iron-sulphur complex formation (Reddy, D. *et al* 1983). Nitrosylated Fe-S complexes have been detected by electron paramagnetic resonance spectroscopy (EPR) in NO-producing macrophages (Lancaster, J. R. Jr. *et al* 1990) and macrophage-injured target cells (Drapier, J.-C. *et al* 1991).

1-4-2-3 Iron loss

Tumor cells that develop cytostasis and inhibition of mitochondrial respiration in response to activated macrophages, release a significant fraction of their intracellular Fe content and this phenomenon does not show in the metabolic inhibitor-treated tumor cells (Hibbs, J. B. Jr. *et al* 1984 ; Wharton, M. *et al* 1988). Activated macrophages inhibit aconitase in the target cells as a result of the loss of an iron atom from the 4Fe-4S cluster within the aconitase active site and aconitase activity of damaged target cells can be restored by incubation with ferrous ion under reducing conditions (Drapier, J. -C. *et al* 1986). Addition of an NO-generating agent "sodium nitroprusside " to the nonheme iron storage protein

ferritin results in the release of Fe and this released Fe catalyses lipid peroxidation (Reif, D. W. *et al* 1990).

1-4-2-4 Inhibition of DNA synthesis and DNA damage

A prominent action of macrophage-derived NO in tumor cells is the inhibition of their DNA synthesis (Krahenbuhl, J. L. *et al* 1974). Interruption of DNA synthesis is not simply a consequence of the inhibition of mitochondrial respiration, one of the potential molecular targets is ribonucleotide reductase (RNR) (Lepoivre, M. *et al* 1990). RNR is a key enzyme, being rate-limiting in the DNA replication process, catalysing the reduction of ribonucleotides into deoxyribonucleotide precursors of DNA, and thereby maintaining and finely regulating the pools of the deoxyribonucleotides. NO quenching of its tyrosyl radical (Lepoivre, M. *et al* 1991), or reaction with its non-heme iron, inhibits the RNR and leads to an interruption of DNA synthesis (Henry, Y. *et al* 1993). Moreover, NO and NO-generating compounds can be oxidized to a mutagen that deaminates DNA. Most DNA sequence changes are C-T transitions, consistent with a cytosine-deamination mechanism (Wink, D. A. *et al* 1991). It also induces dose-responsive DNA strand breakage (Nguyen, T. T. *et al* 1991 ; 1992).

Molecular class	Affected target	Functional effect(s)
Heme proteins	[1] Cytochrome P450	Decrease in cytochrome P450 dependent metabolism
Fe-S Proteins	[2] NADH:ubiquinone oxidoreductase [2] Succinate:ubiquinone oxidoreductase [3] *Cis*-aconitase	Inhibition of mitochondrial respiration
Other non-heme Fe proteins	[4] Ferritin [5] Ribonucleotide reductase	Iron loss Inhibition of DNA synthesis
Tyrosyl radical protein	[6] Ribonucleotide reductase	Inhibition of DNA synthesis
DNA	[7] Cytosine-deamination	Increased mutation and [8] DNA strand breakage
Superoxide anion		[9] Generation of OH• [10] Peroxynitrite

Table 1-1 **Molecular mechanisms of NO cytotoxicity**

1. Khatsenko, O. G. *et al* 1993
2. Granger, D. L. *et al* 1982
3. Drapier, J.-C. *et al* 1986
4. Reif, D. W. *et al* 1990
5. Henry, Y. *et al* 1993
6. Lepoivre, M. *et al* 1991
7. Wink, D. A. *et al* 1991
8. Nguyen, T. *et al* 1992
9. Beckman, J. S. *et al* 1990
10. Denicola, A. *et al* 1993

1-4-2-5 Affect on other proteins

The inactivation of sulfhydryl-dependent bacterial dehydrogenase has long been considered critical to the food preserving properties of acid nitrite. Cytochromes P450 , are a major class of heme-containing proteins which catalyse the oxidative metabolism of endogenous and exogenous substances. NO produced by immunoactivated cell mediates suppression of the enzyme in vitro and in vivo (Khatsenko, O. G. *et al* 1993). Adenosine diphosphate (ADP)-ribosyltransferase, catalyzes the transfer of ADP-ribose from NAD to protein and is activated by NO (Brüne, B. *et al* 1989).

1-4-3 Interaction of NO with other molecules

1-4-3-1 Hemoglobin and Iron inhibition of NO activity

Macrophage-induced cytotoxicity can be blocked by iron (Fe^{2+} and Fe^{3+}), hemoglobin and methemoalbumin (Weinberg, J. B. et al 1977). Both ferrous hemoglobin and methemoglobin remove the NO from the solution, although ferrous hemoglobin is more effective (Martin, W. *et al* 1986). Hemoglobin, which binds nitric oxide, inhibits sodium nitroprusside's (NO donor) cytotoxic activities (Brüne, B. *et al* 1989 ; Dawson, V. L. *et al* 1991), blocks vessel relaxation effect of NO (Palmer, R. M. J. *et al* 1987) and inhibits iron release from ferritin of target cells (Reif, D. W. *et al* 1990). Nitric oxide has an extremely high affinity for the binding site of hemoglobin and binds slowly and reversibly to methemoglobin (Henry, Y. *et al* 1993).

1-4-3-2 Albumin and NO

Although sulfhydryl-containing proteins (eg. albumin) bind with NO readily to form S-Nitroso protein complexes, these complexes still possess the EDRF-like effects of vasodilation and inhibition aggregation of platelets (Stamler, J. S. *et al* 1992), they may serve as a NO carrier molecule (McCall, T. *et al* 1992) Similarly, albumin does not inhibit the tumoricidal effect of activated macrophages (Weinberg, J. B. *et al* 1977).

1-4-4 Other main physiological effects of NO

1-4-4-1 Platelets

Stimulation of endothelial-monolayer cells in culture releases prostacyclins and nitric oxide which are capable of inhibiting the effects of platelet aggregation and adhesion (Radomski M. W. *et al* 1987 a,b,c). There is a synergistic interaction between NO and prostacyclins on anti-aggregation activity (Radomski, M. W. *et al* 1987 c) but no such potentiation on anti-adhesion. NO has a role in determining the non-adhesive properties of the vascular endothelium by a cGMP mediated mechanism (Radomski, M. W. *et al* 1987 a). Nitric oxide synthase, is present in human platelets and is dependent on NADPH, free intracellular calcium, inhibited by L-NMMA. It is stimulated to enhance NO synthesis from L-arginine once platelet aggregation is initiated. NO generated from platelets acts as a negative feedback system to modulate aggregation (Radomski, M. W. 1990).

1-4-4-2 Central Nervous System

Nitric oxide is a central and peripheral neuronal messenger. It is involved in classical antegrade neuronal signalling and also has unique properties as a retrograde transmitter. In the central nervous system the excitatory neurotransmitter glutamate can elicit large increases in cGMP levels and releases a diffusible messenger which involves cell-cell interaction (Garthwaite, J. *et al* 1987). This messenger is identified as having properties identical to NO (Garthwaite, J. *et al* 1988 ; Bredt, D. S. *et al* 1989). Stimulation of the glutamate-related amino acid N-methyl-D-aspartase (NMDA) receptor has been shown to activate synthesis of NO postsynaptically and it does not act on the generator cells but diffuses out to act on one or more neighbouring structures including presynaptic terminals and astrocytes. NO is also formed presynaptically in climbing fibres of the cerebellum. The effect of NO generated in these fibres is the long-term depression of responses in Punkinje fibres since exogenous NO causes, and L-NMMA blocks, this depression (Shibuki, K. *et al* 1991). A second function for NO within the CNS may relate to it's toxic effects. NADPH diaphorase positive neurons are resistant to this toxic insult. Because cNOS is one NADPH diaphorase, it is possible that cNOS-containing neurons kill cNOS-negative cells (Dawson, V. L. *et al* 1991). Selective neurotoxicity may be important during brain development and NO-releasing neurons could contribute to this process (Bredt, D. S. *et al* 1991).

1-4-4-3 Peripheral nervous system

In the peripheral nervous system, NO fulfils many of the criteria of a classical neurotransmitter although it is not released in quanta. Inhibitory non-adrenergic, non-cholinergic (NANC) nerves are thought to be important in the autonomic innervation of the gastrointestinal tract and other organ systems. NO could account for the biological activity of this transferable NANC factor (Bult, H. *et al* 1990). In the stomach, vagal stimulation releases NO to relax gastric smooth muscle (Desai, K. M. *et al* 1991). In the vasculature, NO released from NANC nerves mediates vasodilatation directly and modifies transmitter output.

1-4-4-4 Vascular

The endothelium contributes to the regulation of vascular tone by various mechanism. One is release of a powerful vasodilator, 'endothelium-derived relaxing factor' (EDRF) (Furchgott, R. F. *et al* 1980). EDRF is a labile humoral agent which mediates the relaxation of vascular smooth muscle via the elevation of cGMP levels (Moncada, S. *et al* 1988). Vascular endothelial cells in culture synthesize NO from L-arginine (Palmer, R. M. J. *et al* 1988a), L-NMMA inhibits this synthesis in a dose dependent and enantiomerically specific manner (Palmer, R. M. J. *et al* 1988b). The co-product of this reaction is L-citrulline (Palmer, R. M. J. *et al* 1989). NO released from bradykinin-stimulated endothelium is indistinguishable from EDRF in terms of it's biological activity, stability and susceptibility to an inhibitor, or to a potentiator (Palmer, R. M. J. *et al* 1987 ; Hutchinson, P. J. A. *et al* 1987).

Constitutive NO synthase in the endothelial cells provides a locally regulated mechanism releasing NO continuously in varying amounts to regulate blood vessel tone (Moncada, S. *et al* 1991). A similar, tightly regulated physiological vasodilator influence is provided by NO released from NANC nerves in the adventitia of blood vessels. In the penis, NO released from nerves leads to relaxation of the corpus cavernosum and may contribute to erection in animals and humans (Holmquist, F. *et al* 1991 ; Ignarro, L. J. *et al* 1990).

1-4-4-5 Other systems

NO regulates the microcirculation of rat kidneys. It may also play a role in the control of tubuloglomerular feedback and renin secretion (Nathan, C. 1992). The pulmonary vasoconstriction due to hypoxia or pulmonary hypertension, is reversed by NO and it also protects against adult respiratory distress syndrome (Moncada, S. *et al* 1992). NO inhibits the proliferation of lymphocytes which respond to mitogens or antigens, and prevents the adhesion of leukocytes to endothelium. Both cNOS and iNOS may participate in the control of insulin release (Nathan, C. 1992). Possible functions of NO are outlined in table 1-2.

TABLE 1-2 **The possible functions of NO**

Systems/Cells	Possible function(s)
Vascular	Vessel dilatation
Nervous	Neuronal messenger Neurotoxicity
Macrophages	Cytotoxicity
Platelets	Decreases aggregation and adhesion
Renal	Regulates microcirculation Determines renin release
Pulmonary	Reverses pulmonary vasoconstriction
Immunology	Inhibits lymphocyte proliferation
Encdocrine	Participate insulin release

Information summary from Moncada, S. *et al* 1992 and Nathan, 1992.

1-4-5 NO synthases

NO is formed in the generator cells, from arginine by a nitric oxide synthase and is associated with stoichiometric production of citrulline. Many different isoforms of nitric oxide synthase (NOS) have been characterized and purified from different cell types (Moncada, S. *et al* 1991; Nathan, C. 1992; Förstermann, U. *et al* 1991; Lyons, C. R. *et al* 1992). Among the cytosolic nitric oxide synthases, two main classes can be distinguished. The brain and endothelial enzymes are both constitutive, whereas the macrophage

NOS is inducible. Both classes of NOS are nicotinamide adenine dinucleotide phosphate (NADPH) dependent. Structural analogs of arginine such as N-monoethyl-L-arginine (NMMA) are strong, although not absolutely specific, inhibitors of NOS and permit detection of this enzymatic reaction in intact cells, organs, or animals (Palmer, R. M. J. *et al* 1989; Bredt, D. S. *et al* 1990; Marletta, M. A. 1988; Gross, S. S. *et al* 1990). Molecular oxygen is incorporated into both NO and citrulline by both classes NOS, thus they are also dioxygenases (Kwon, N. S. *et al* 1990 ; Leone, A. M. *et al* 1991). Both rat brain and murine macrophage NOS have been recognized as a CO-binding cytochrome P450 type hemoprotein (Bredt, D. S. *et al* 1991 ; Lowenstein, C. J. *et al* 1992). The constitutive enzyme is Ca^{2+}/calmodulin dependent (Busse, R. *et al* 1990), and releases NO for short periods in response to receptor or physical stimulation. The NO released by this enzyme acts as a transduction mechanism underlying several physiological responses and its molecular target is guanylate cyclase (GC), a hemoprotein. The activation of GC leads to increased levels of cGMP and to a cascade of molecular events within the target cells (Moncada. S. *et al* 1991). The inducible enzyme is Ca^{2+}/calmodulin independent (Stuehr, D. J. 1991). It requires *de novo* protein synthesis for its expression (Xie, Q.-W. *et al* 1992) and the activity is not detectable in either macrophage cell lines or freshly elicited macrophages that have not been activated (Marletta ,M. A. *et al* 1988 ; Stuehr, D. J. *et al* 1987a). It is released over long periods and it's induction is inhibited by glucocorticoids (O'Connor, K. J. *et al* 1991). The properties of these two classes of NOS are listed in table 1-3.

TABLE 1-3 **Similarities and differences between the two main classes of NOS**

Constitutive [1.]	**Inducible** [2.]
[3.] NADPH dependent [2.]	
[4.] Dioxygenase [5.]	
[6.] P450 type hemoprotein [7.]	
[3.] Inhibited by L-arginine analogs [2.]	
Ca^{2+}/calmodulin dependent [8.]	Ca^{2+}/calmodulin independent [9.]
Short-lasting release [10.]	Long-lasting release [11.]
Unaffected by glucocorticoids [10.]	Induction inhibited by glucocorticoids [11.]

1. Janssens, S. P. *et al* 1992
2. Marletta, M. A. *et al* 1988
3. Palmer, R. M. J.. *et al* 1989
4. Leone, A. M. *et al* 1991
5. Kwon, N. S. *et al* 1990
6. Bredt, D. S. *et al* 1991
7. Lowenstein, C. J. *et al* 1992
8. Busse, R. *et al* 1990
9.Stuehr, D. J. *et al* 1991
10. Moncada, S. *et al* 1991
11. Xie, Q.-W. *et al* 1992

1-4-6 Roussin's black salt

Roussin's black salt (RBS) belong to a class of compounds which has been known for over a century (Roussin, F. Z. 1858). The chemistry of this compound is unique, it arises by a process known as "spontaneous self assembly" (Sung, S.-S. *et al* 1985). That is to say, under appropriate conditions, synthesis occurs from mononuclear starting materials without the detectable formation of any intermediates. In the solid state, the compound is relatively stable,

particularly if kept under nitrogen and at -10°C. Once in solution it will decompose to release NO and produces iron oxide and elemental sulphur, although it is unclear whether other intermediates exist (personal communication with Dr. I Megson). RBS, contains seven nitrosyl groups linked to an iron-sulphur framework and relaxes the arterial muscle of a small segment of pre-contracted rat tail artery. The vasodilatory effect of RBS is blocked by methylene blue, an inhibitor of the soluble guanylate cyclase, and by ferrous hemoglobin, a NO scavenger (Flitney, F. W. *et al* 1990). The release of NO from RBS is accelerated during exposure to laser light (λ=514.5 and 457.9 nm) (Flitney, F. W. *et al* 1993). NO inhibits the proliferation of lymphocytes which response to mitogens or antigens (Nathan, C. 1992), although this inhibition is independent of the source of NO. RBS causes the increased inhibition of lymphocyte proliferation, with increasing nitrite concentrations in the culture supernatant (Rowland, I. J. *et al* 1991).

Purpose of present studies

In the scale of tumor incidence bladder tumors rank high especially in industrialized areas. In view of the fact that the best way to treat a disease is to stop it before it starts to develop, it is thus important to understand the tumorigenesis of human urothelium tumors for the purpose of prevention. A normal human urothelium cell line (SV-HUC-1 although theoretically speaking not a normal cell because of its immortality) was used for tumorigenic transformation experiments. Furthermore experiments were carried out to define

any molecular changes associated with this transformation by assaying medium conditioned by parent cells and transformed cells. For the treatment of established bladder cancers or prophylactic management of recurrences, local instillation of chemotherapeutic agents into the patient's bladder was performed with limited success and a wide range of complications. An ideal drug has yet to be found and undergo trail. NO is a natural product with cytotoxic effect of the human body. To further probe the therapeutic role of NO, a novel NO donor (RBS), was assayed for its cytotoxicity on both non-tumorigenic (SV-HUC-1) and tumorigenic human (T24) urothelium cells. Attempts to identify the possible mechanism of this growth inhibition effect were carried out using hamster ovary cell(CHO) and its radiosensitive mutant (xrs-5).

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2-1 Cell lines and cultures

2-1-1 SV-HUC-1 cell line

SV-HUC-1 is a normal human urothelial cell line which was immortalised with Simian virus 40 (SV40). It has an unlimited life span and currently following at least 50 passage of these cells has remained nontumorigenic as determined by their inability to form tumors in athymic nude mice. Cultures are epithelial in appearance and show some morphological heterogeneity in cell size and shape. Positive immunohistochemical staining for human cytoplasmic keratin and T antigen of SV40 has been demonstrated. (Christian, B. J. *et al* 1987).

Materials

Ham's F12 [Gibco, Grand Island, NY] supplemented with 7% (v/v) fetal calf serum (FCS) [Globepharm, U.K.] This medium, referred to as "7% FCS F12", also contained 100 units/ml Benzyl penicillin, 100 μg/ml streptomycin [Sigma, USA] and 2 mM L-glutamine [Flow laboratories, U.K.]. Disodium ethylenediaminetetraacetic acid (EDTA) [Sigma] in Hanks's balanced salt solution (HBSS).

Cell culture

Cells were grown in 80cm^2 flask [Nunc,Denmark] with 10 ml medium and gassed with 5% CO_2 and air to reach an optimal pH. Caps were screwed air tight and flasks were kept in a 37°C dry incubator receiving a complete medium change 3 times a week.

Passage

All the medium was removed and the flasks rinsed with 10mls of 0.1% (w/v) EDTA in HBSS. Flasks were placed in a 37°C incubator for 20 minutes with fresh EDTA solution and the cells detached by tapping the flask gently. An equal volume of 7% FCS F12 medium

was added to the flask then all the fluid transfered into a 30 ml universal tube [Sterilin, U.K.]. The fluid was centrifuged at 1000 rpm for 10 minutes to obtain a cell pellet, supernatant removed and cells resuspended in medium by gentle pipetting. The cellularities were determined using a hemocytometer (Reznikoff, C. A. *et al* 1983).

2-1-2 T24 cell line

T24: This cell line was originally called "MGH-U1", but it's HLA phenotypes (O'Toole, C. M. *et al* 1983) and DNA fingerprinting pattern (Master, J. R. W. *et al* 1988) are identical to the T24 cell line which is derived from a human urinary bladder carcinoma. It will produce a tumor following inoculation of cell suspension into the cheek pouch of a hamster (Bubenik, J. *et al* 1973).

Cell culture

The method of culturing this cell line was identical to that used for the "HUC-1" cells [2.1.1] with the exception that Rosewell Park Memorial Institute tissue culture medium 1640 (RPMI-1640)[ICN biomedicals UK] supplemented with 5%(v/v) FCS also contained 100 units/ml Benzyl penicillin 100 µg/ml streptomycin and 2 mM L-glutamine was used.

Passage

The medium was removed from flasks. 5mls of 0.05%(w/v) trypsin [Difco UK] in 0.2%(w/v) EDTA and PBS solution (PE) were added and swirled gently across the cells then removed and replaced with fresh trypsin. This was removed and a thin layer of trypsin was left in the flask. Flasks were kept in a 37°C incubator for 5 minutes until cells were observed to round up. Flasks were gently shaken to release the cells and medium added then pipetted gently to make a

single cell suspension. Cells were counted with a hemocytometer and passaged into a new flask.

2-1-3 CHO and xrs cell lines

CHO-K1 Chinese hamster ovary cell line

xrs-5 Radiation-sensitive mutant, which is DNA repair deficient, isolated from CHO-K1 (Jeggo, P. A. *et al* 1983).

Cell culture

The method of culturing these cell lines was identical to those used for the "HUC-1" cells [2-1-1] with the exception that Eagle's minimal essential medium [Gibco F15] supplemented with 10% (v/v) foetal calf serum also contained 100 units/ml Benzyl penicillin 100 μg/ml streptomycin and 2mM L-glutamine was used.

Passage

The method of passaging these cell lines was identical to that used for the "T24" cells [2-1-2] with the exception that 0.05%(w/v) trypsin with 0.7mM EDTA in PBS (TE) were used for detaching cells.

2-2 Cryopreservation and rederivation of cell lines

2-2-1 Cryopreservation

The cells were harvested and centrifuged. Cell pellets were resuspended in culture medium to a concentration of $2x10^6$cell/ml and a one tenth volume of dimethyl sulphoxide (DMSO) [BDH] added slowly and equilibriated by gently swirling. 1 ml of mixture was aliquoted into a cryotube [Nunc] and frozen slowly in the gas phase of a liquid nitrogen freezer for at least 24 hours before immersion into the liquid phase (-196°C).

2-2-2 Rederivation

Cryotubes were carefully removed from liquid nitrogen, the cell suspension were thawed rapidly in a 37°C water bath and the cells put into a flask. Culture medium was added drop by drop slowly, flasks gassed with 5% CO_2 and air and kept in a 37°C incubator. A change of medium was made after there was evidence of cell attachment.

2-3 SV-HUC-1 cells growth in serum free, factors free (sff), chemically defined medium

2-3-1 Aim and principle

Aim: Isolation of human urothelium cell variants, called "autotrophs" which are able to grow using low molecular weight metabolite precursors and in the absence of macromolecules and serum replacement factors (Hillova, J. *et al* 1990).

Principle: Using a selection procedure which switches culture conditions of the cells from a 7% FCS supplemented medium to a medium without addition of exogenous growth factors, hormones, or serum.

2-3-2 Materials and methods

Cells: SV-HUC-1 [2-1-1].

Ham's F12 medium added with 100U/ml penicillin, 100µg/ml streptomycin and 2 mM L-Glutamine this referred as "sff F12" 0.05%(w/v) soyabean trypsin inhibitor [Sigma] in PBS. 0.1%(w/v) EDTA in HBSS, 0.05%(w/v) Trypsin in PE.

Cell culture

SV-HUC-1 cells were harvested as described [2-1-1] but the cell pellet was resuspended in sff F12 medium and acquired number of cells were put into 80 cm^2 flasks [Nunc] containing 10 mls of sff F12 medium, gassed with 5% CO_2 to optimal pH, cap closed air tight and flasks kept in a 37°C dry incubator. Once cells attached to the flask a complete medium change was received every second day. These surviving and proliferating cells were designated as "SV-HUC-1N".

Passage

Cells were grown to confluence in flasks, medium was removed, and cells rinsed with 0.1% EDTA in HBSS. 2.5 mls of 0.1% EDTA and 2.5 mls of 0.05%(w/v) trypsin in PE were added for 3 minutes and removed. 5 mls of 0.05%(w/v) soyabean trypsin inhibitor in PBS were added for 3 minutes and removed. Flasks were kept at 37°C for 20 minutes with 10 mls of 0.1% EDTA in HBSS. Cells were detached by tapping the flask gently and an equal volume of sff F12 medium was added before all the fluid was transfered into a 30 ml universal tube [Sterilin]. The fluid was centrifuged at 1000 rpm for 10 minutes to obtain a cell pellet, supernatant removed and cells resuspended in sff F12 medium by gentle pipetting. Cellularities were determined with a hemocytometer.

2-4 Assay of cellular tumorigenicity

2-4-1 Aim and principle

Aim: Determination the tumorigenicity of cells.

Principle: Inoculation of cells into congenitally immuno-deficient athymic nude mice will lead to the development of tumors if the cells are tumorigenic.

2-4-2 Materials and methods

Nude mice were used [nu/nu, MF1, Olac, U.K]. Mice kept in the University of St. Andrews Animal Handling Facility in a 12 hour light/dark illumination cycle, at an ambient temperature of 22.3°C (72°F) and in which the air was filtered. Mice were fed with RM1(E) pellets [Special Diet Service, U.K.] and allowed sterile water *ad libitum* .

The cells were harvested and cellularities determined as described previously. The cell pellet was resuspended in a minimum amount of medium (< 500 µl) and the medium containing cells was sucked into a 1 ml syringe fitted with a 21 gauge needle. The dorsal aspect of the neck of the nude mice was chosen as the injection site. The skin was sterilized with a 70%(v/v) alcohol swab and the subcutaneous inoculation of cells performed. Mice were kept in the animal house and injection sites were observed twice weekly. Tumor growth was monitored until autopsy at a suitable size. In the case of no tumor growth mice were monitored for 6 months.

2-4-3 Tumor dissection and tissue explant culture

Aim: Growing tumor cells out from the tumor tissue.

Method

Mice with tumors were killed by cervical vertebrae dislocation. The tumors were excised using sterile technique and put into a petri dish containing medium. Excessive connective tissue was removed and a slice of tissue was cut out from the middle of tumor then further cut into $1mm^3$ size fragments. The fragments were placed

in a 25cm^2 flask with 7% FCS F12 medium sufficient to wet the bottom of the flask. Once the cells were confluent they were cultured and passaged as previously described [2-1-1]. These surviving and proliferating cells were designated as "SV-HUC-1T".

2-5 Colony formation in semi-solid methylcellulose

2-5-1 Aim and principle

Aim: Cloning of cells.

Principle: Cells form colonies in the semisolid methylcellulose. These colonies can be aspirated from the methylcellulose and regrow in culture flasks as a subclone. (Mcniece, I. K. *et al* 1986).

2-5-2 Materials and methods

Methylcellulose [Fluka, Germany] was prepared in advance. 2.145 gms of methylcellulose and a magnetic stirrer bar were autoclaved together in a beaker. 50 mls of boiling Milli-Q water were added and the solution stirred continuously using a magnetic stirrer [Gallenkamp U.K.]. Once cooled, 50 mls of double strength Ham's F12 medium were added. The mixture was kept at +4°C with constant stirring overnight. (A clear gel like solution should be produced by the next morning or it should be discarded). 20 mls of solution were aliquoted into universal tubes, capped,covered with tin foil and kept at -20°C until required.

Feeder layer

9 mls of 10%(v/v) FCS F12 medium and 1 ml of melted 5%(w/v) agar [Bacto-Aga, Difco, U.S.A.] were mixed together and 2 mls of this preparation aliquoted into a 60 mm diameter plastic triple vent petri dish [Nunc] and allowed to set.

Cellular layer

Cells were harvested as described previously [2-1-1] and a required number of single cells were suspended in 4.5 mls of 10% FCS F12 medium. This was added to 4.5 mls of 37°C, 2%(w/v) methylcellulose and mixed well. 2 ml aliquots were plated on top of each feeder layer and dishes were kept in a 37°C, 5% CO_2 in air, fully humidified incubator for 21 days. Single colonies which were identified using a dissecting microscope set up to give dark field illumination [Olympus, Japan] were removed from the methylcellulose under sterile conditions using a 21 gauge needle connected to a 1 ml syringe containing 0.2 ml of 10% FCS F12 medium. Cells were put into a 25cm^2 flask [Nunc] with 1 ml of medium, gassed with 5% CO_2, cap closed air tight and flasks kept in a 37°C dry incubator. 5 mls of medium were added once the cells were attached to the flask and medium changed three times a week. These surviving and proliferating cells were designated as "SV-HUC-1TS".

2-6 Chromosome studies

2.6.1 Aim and principle

Aim: Distinguishing between human and murine cells.

Principle: Observation of chromosomes at metaphase in the cell cycle when they are most easily recognized.

2-6-2 Materials and methods

Glass slides (76x26 mm) [BDH] were prepared in advance by immersion in 1M hydrochloric acid (HCl) overnight and rinsing with water. These clean glass slides were immersed in 100%(v/v)

ethanol overnight and air dried. The slides were kept in a clean box and cooled to -20°C before use.

Cells in metaphase were accumulated by adding 0.04µg/ml of colcemid (stock solution: 4µg/ml) [Sigma] to cell cultures, 3-4 hours before harvesting. Hypotonic treatment of harvested cells was performed by resuspending the cell pellet in 75 mM KCl. After 3 minutes at room temperature cells were centrifuged and supernatant removed. The cell pellet was dispersed by flicking the tube and the cells were fixed in fresh Carnoy's fixative (Methanol:Glacial acetic acid = 3:1). This procedure was repeated at least 3 times by centrifugation and resuspension in Carnoy's fixative. (The cells can be stored in the fixative at +4°C for several months.) For chromosome spreads, these cells were dispersed in a few drops of fresh fixative, dropped onto -20°C chilled slides and immediately warmed by hand. Slides were rapidly dried by heat and stained with 3%(v/v) Giemsa [BDH] in water for 20 minutes. Stained preparations, which were rinsed with water and air dried, were then examined under oil immersion by light microscopy [Leitz wetzlar, Germany].

2-7 Immunocytochemical staining of cells and tissue

2-7-1 Aim and principle

Aim: Identification of a specific antigen of the target cells thereby establishing the origin of the cells.

Principle: A primary antibody binds to the cellular antigen of interest and this antigen-antibody interaction is further amplified by the introduction of a secondary peroxidase-conjugated antibody

which binds to the primary antibody. The peroxidase is then visualised using the enzyme reaction with 3,3-diaminobenzidine (DAB) in the presence of H_2O_2. This reaction gives a brown staining deposit that can be observed with the light microscope.

2-7-2 Materials and methods

Primary antibodies:

DAKO-M821: A mouse monoclonal antibody which reacts with an epitope which is present in a wide range of human cytokeratins was used. A positive stain indicates the target cells are human epithelial in origin. Used in a 1:25 dilution in 1%(w/v) bovine serum albumin in PBS (BSAPBS).

PAb 405: A mouse monoclonal antibody which reacts with a region of the SV40 T- antigen close to the C-terminus. Positive stainings may indicate the target cells are immortalized by SV40.

DO-1: A mouse monoclonal antibody which reacts with human, monkey, bovine mutant and wild type p53. Epitope in N-terminus region aa 1-45.

Secondary antibody:

DAKO-P161: A rabbit anti-mouse IgG, Horseradish peroxidase (HRP) conjugated, polyclonal antibody. diluted 1:100 in BSAPBS.

DAKO-M821 and *DAKO-P161* were obtained from DAKOPOTTS, Denmark. *PAb 405* and *DO-1* were obtained from Professor D. Lane, Dept of Biochemistry, University of Dundee. Used neat in the form of hybridoma culture supernatant, or ascites fluid, diluted 1:500 in PBS (Vojtesek, B. *et al* 1992).

50 µls of $1x10^6$ cells/ml single cell suspension was dropped on one end of a glass slide which had been sterilized in advance. These preparations were kept in a humid chamber and incubated in a 37°C, 5% CO_2, humidified incubator overnight. Slides were rinsed

once with PBS and fixed in cold acetone for 20 minutes. After air drying the slides were flooded with 1%(w/v) BSAPBS and kept at 37°C in a humid chamber for 60 minutes to block the nonspecific binding of antibodies. A primary antibody was added to the cells and kept in a humid chamber at 37°C for 60 minutes. After rinsing the slides 3 times with BPS to remove the un-bound primary antibody, the secondary antibody was added. Slides were incubated, and the secondary antibody washed off, as detailed above then developed with 3',3-diaminobenzidine (DAB) [Sigma]. DAB was used at a concentration of 1 mg/ml in water with 0.03%(w/v) nickle sulphate (to enhance the black coloration) and activated with 0.06% H_2O_2. Slides were kept in developing solution for 5-10 minutes in the dark then rinsed with water. The protocol in all cases remained essentially the same with the exception that no further fixation was required in the wax embedded tumor tissue slides which were dewaxed in hot sterile water prior to staining.

2-8 DNA finger printing

2-8-1 Aim and principle

Aim: Identifying the parental origin of the cells.

Principle: Cellular genomic DNA was cut with restriction endonuclease, separated by electophoresis, and hybridized with probe. Comparison between the patterns of these small fragments was performed to find evidence of genetic linkage and mutation.

2-8-2 Materials and methods

Detailed methods and materials can be found in the Appendix I. A summary of the whole procedure is presented here.

2-8-2-1 Extraction of the genomic DNA

Approximately $1.5x10^7$ cells were harvested using trypsin or EDTA and washed 3 times in PBS. Cells were dispersed in a 1.8 mls Eppendorf tube containing 1 ml of lysis buffer,100 µls of 10 mg/ml proteinase K, 250 µls of 10%(m/v) SDS and incubated at 50°C with gentle shaking overnight. DNA was extracted from lysate by the addition of an equal volume of phenol(x1), chloroform/phenol(x2), chloroform(x1) and precipitated in 2 volumes of ice cold 100% ethanol and a half volume of 7.5M ammonium acetate. DNA was rinsed twice with 70% ethanol, dried in air, and dissolved in 100µls of TE.

The quality of DNA was checked by electrophoresis in a 0.8%(w/v) agarose gel with TBE as electrophoresis buffer at 80 volts for 45 minutes. The quantity of DNA was measured by a DNA fluorometer [TDK 100, Hoefer Scientific Instrument, USA] and adjusted to a concentration of 250-500 µg/ml in TE.

2-8-2-2 Digestion of DNA with restriction endonuclease

60 µls of sample DNA was cut by the addition of 4 µls of endonuclease HaeIII (12u/µl), 8 µls of (10x)HaeIII buffer and 8 µls of 0.1%(w/v) BSA and incubated at 37°C overnight. The digested DNA was extracted by the addition of an equal volume of phenol/chloroform (x1), chloroform (x1) and purified in ice cold 100% ethanol with 2.75M sodium acetate, before dissolving in 20 µls of TE.

2-8-2-3 Separation of the DNA fragments by agarose gel electrophoresis

All the DNA samples and a lambda ladder marker were run in a 0.8%(w/v) agarose gel with TBE as electrophoresis buffer at 24 volts for 48 hours, then run at 36 volts, after running buffer was

changed, for another 24 hours. DNA fragments were separated on the agarose gel and denatured *in situ*. The fragments were then transferred from the gel to a solid support (membrane), where they were immobilized. After prehybridization to reduce nonspecific hybridization by the probe, the membrane was hybridized to the desired radiolabeled nucleic acid probe. The membrane was washed to removed unbound and weakly binding probe, and was then autoradiographed.

2-8-2-4 Transferring DNA to a membrane by Southern blotting

The gel was immersed in 0.25 N HCl for 20 minutes to depurinate the DNA, in GS1 solution for 45 minutes to denature the DNA and in GS2 solution for 45 minutes to neutralize the gel. The DNA fragments on the gel were transferred to a nitrocellulose membrane [Zeta-Probe GT blotting membrane, Bio-RAD] by the standard Southern blotting procedure with (x10)SSC as the transferring buffer. Briefly a sheet of nitrocellulose membrane was put on top of the gel which was placed on a Whatman 3 MM paper wick tray. Two sheets of Whatman 3 MM paper with a pile of absorbant paper were put on top of the membrane to keep up a steady capillary pressure. Damp absorbant paper was replaced several times in the first hour then the transfer pyramid left overnight. Membrane was washed with (2x)SSC and baked at 80°C in a vacuum oven for 30 minutes.

2-8-2-5 Production of the probe and hybridization with the membrane

The probe was a segment of ^{32}P radiolabeled RNA which was synthesized from a specific DNA template inserted down stream from a very specific bacteriophage transcription promoter. The pSPT 18.15 was used as a vector and linearlized with the

restriction enzyme HindIII. Transcription reaction was carried out with T7 RNA polymerase and stopped by the addition of nick stop mix. The unincorporated nucleotides were removed by spin column chromatography through a 1 ml column of Sephadex G50 equilibriated with TE. The radioactivity of the probe was measured with a liquid scintillation analyser. The membrane was prehybridized with a solution containing 1% BLOTTO(w/v), 2%(w/v) SDS, and (x1)SSC at 64°C overnight. $6x10^7$ cpm RNA probe was added to the same chamber and hybridized at 65°C overnight. The membrane was washed in a shaking water bath at 65°C for 45 minutes with 3 changes of wash solution containing (x1)SSC, 0.1%(w/v) SDS and wrapped in Saranwrap after it had been air dried.The membrane was exposed to X-ray film and kept at -70°C for 2 days with two intensifying screens followed by 14 days without screens. Films were developed and DNA fragments compared.

2-9 Assay of cellular DNA content by flow cytometry

2-9-1 Aim and principle

Aim: Comparison of the distribution of DNA content in tumorigenic and non-tumorigenic cells.

Principle: A DNA specific dye is employed to stain cells and the amount bound is proportional to DNA content. Individual cells are then directed through a laser beam to excite the dye, and the fluorescence emission is collected and displayed as a fluorescence distribution. This will enumerate the cells containing different amounts of DNA (Barlogie, B. *et al* 1983).

2-9-2 Materials and methods

Cells were obtained as previouly described [2-1-1], fixed in 70%(v/v) ethanol and kept in +4°C overnight. Ethanol was removed and cells were washed once with PBS by centrifugation and resuspension. Cells were resuspended at $1x10^6$ cells/ml in a 1mg/ml fresh solution of ribonuclease A (RNase A) [Northumbria Biologicals U.K.] in PBS and incubated at 37°C for 30 minutes. Propidium iodide (PI) [Sigma] was added to the cells to a final concentration at 4 μg/ml. Cells were dispersed through a 21 gauge needle. Data for the measurements of forward scatter versus fluorescence of PI were acquired in a flow cytometer [FACScan, Becton Dickinson USA.] using a software package "Consort *30", and the populations of the cells with different DNA content were analysed using "DNA" software.

2-10 Measurement of doubling time of HUC-1T cells

A given number of cells were plated in several 25 cm^2 flasks [Nunc] containing culture medium (7% FCS F12). They were gassed with 5% CO_2, caps closed air tight and kept in a 37°C incubator. Cells were harvested and counted at regular intervals. Starting 48 hours after plating using 0.05% trypsin in PE. Cell counts and viabilities were determined on cells stained in 2%(m/v) fast green using a hemocytometer as described in [2-16-3] and data used to plot growth curve (Gioanni, J. *et al* 1985).

2-11 Vital stain of cellular foci

2-11-1 Aim and principle

Aim: Demonstration of the living cells making up the foci formed in culture flask.

Principle: Using confocal microscope to show the fluorescein in the living cells.

2.11.2 Materials and methods

SV-HUC-1TS cells were grown in 25 cm^2 flask as previously described until foci formed. Flasks were rinsed 3 times with PBS, a mixture of 15 µl of a stock solution of 5-carboxyfluoresein diacetate (5 C-FDA) [Sigma] (10mg/ml in AnalaR acetone) and 5 mls of PBS was added to the flask. Flasks were incubated at 37°C for 10 minutes, supernatant removed and flasks rinsed once with PBS. Cells in flasks were observed using a laser scanning confocal imaging system which were operated in conjunction with a Nikon diaphot inverted fluorescence microscope [BioRad, MRC 600 USA], 488nm light as a illuninating source (Bruning, J. W. 1980).

2-12 *In vitro* murine high proliferative potential colony-forming cell (HPP-CFC) assay

2-12-1 Aim and principle

Aim: Qualitative demonstration of the presence of cytokines in medium conditioned by HUC-1 and HUC-1N cells.

Principle: Based on the ability of certain cytokines to interact with other hematopoietic growth factors to stimulate the proliferation of

HPP-CFC from murine bone marrow in double layer, semisolid agar culture (McNiece, I. K. *et al* 1986).

2-12-2 Materials and Methods

Production of condition medium: Cells were grown to confluence in 10 ml of 7% FCS F12 (for HUC-1) or sff F12 (for HUC-1N) medium in 80 cm^2 flasks. Medium was completely removed and replaced by 10 ml of fresh medium and cells cultured for a further 3 days. Medium was harvested and centrifuged at 3000 rpm for 15 minutes, aliquoted and frozen (-20°C) until required.

Recombinant murine granulocyte-macrophage colony stimulating factor (rmu GM-CSF) [Immunex U.S.A.] used at an optimal concentration of 40U/ml.

Recombinant human macrophage colony stimulating factor (rhu M-CSF) [Genetic Institute U.S.A.] used at 50U/ml.

Recombinant murine stem cell factor (rmu SCF) [Immunex] used at 10 ng/ml.

Murine bone marrow cells were prepared from 8-10 weeks old CBA/H mice which were killed by cervical dislocation. Femora were dissected under sterile condition, femoral joints were transsected and a 1ml syringe containing medium with a 23 gauge needle inserted into the epiphyseal cartilage of the knee joints. Bone marrow cells were flushed out from the femoral cavities and a single cell suspension produced by gently drawing the cells through a 23 then a 25 gauge needle. Cellularities were determined with a Coulter counter [ZM, Coulter Electronics, U.K.].

This assay uses a combination of hematopoietic CSF and employs a bilayer agar culture technique. A feeder layer containing CSFs underlies a cellular layer. CSFs diffused from the feeder layer to

influence the proliferation and differentiation of cells within the upper layer.

Underlayer

A combination of CSFs were added to a volume of 37°C Dulbecco's medium [Gibco, U.K.] containing 20%(v/v) horse serum (HS) [Globepharm, U.K.] sufficient to produce a total of 9 mls. 1 ml of 5% (w/v) melted agar [bacto Agar, Difco, U.K.] was added and 2 mls of this preparation were aliquoted into 60 mm diameter plastic triple vent petri dishes [Nunc]. The agar was allowed to set and these feeder layers were stored in a 37°C ,5% CO_2 in air, fully humidified incubator until required.

Cellular layer

1ml of $2x10^5$/ml of murine bone marrow cells and 1 ml of 3%(w/v) melted agar were added to 8 mls of 37°C Dulbecco's medium containing 20%(v/v) HS. 2 ml aliquots of this mixture were plated on top of the previously prepared feeder layer. The agar was allowed to set and incubated in 37°C, 5% CO_2 in air, fully humidified incubator for 14 days.

Assay of the colonies

12 to 24 hours prior to counting 1 ml of autoclaved solution of 1mg/ml in normal saline of 2(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT)[BDH, U.K.] was added to each culture dish and incubation continued. As viable cells proliferate *in vitro* they convert the colourless tetrazolium salt to a water insoluble red formazan which precipitates inside the cells (Bol, S. *et al* 1977).

2-13 Enzyme linked immuno-sorbant assay (ELISA) for human stem cell factor in medium conditioned by HUC-1 and HUC-1T cells

2-13-1 Aim and principle

Aim: Quantitative determination of human stem cell factor (SCF) in the conditioned medium.

Principle: Monoclonal antibody specific for SCF immobilizes the factor in the conditioned medium. A second enzyme-linked polyclonal antibody binds to the factor and a colour reaction is developed in proportion to the amount of SCF bound in the initial step.

2-13-2 Materials and methods

Conditioned medium was prepared as previously detailed [2-12-2] using 7% FCS F12 medium for both SV-HUC-1 and SV-HUC-1T cells.

ELISA kits used were commercially available. [BIOTRAK, Amersham U.K.], [Quantikine, R&D system U.S.A.].

Procedure as *per* manufactures instructions. Briefly, samples were added to the test microtitre wells which were precoated with monoclonal murine antibody against SCF. Bound SCF was measured using polyclonal SCF antibody conjugated to horseradish peroxidase. A curve of optical density versus the concentration of SCF was generated using standard recombinant human SCF. Concentration of SCF in the sample was determined by comparing the optical density of the samples to this standard curve. The assay range for human serum SCF concentration is 31.3pg/ml to 2000pg/ml.

2-14 Murine thymocyte proliferation assay

2-14-1 Aim and principle

Aim: The detection of interleukin one(IL1)-like activity.

Principle:

1. A subpopulation of thymocytes (T lymphocytes) exposed to IL1 express interleukin two (IL2) receptors.

2. A subpopulation of thymocytes interact with a suboptimal levels of mitogen (Concanavalin A) and release IL2.

3. IL2 stimulated IL2-receptor expressing cells undergo proliferation.

4. Thymocyte proliferation is detected by tritiated thymidine incorporation, and is related to the level of IL1 (Krakauer, T. *et al* 1982).

2-14-2 Materials and methods

Preparation of murine thymocytes: Thymocytes were harvested from the 2-3 weeks old CBA/H mice which were killed by cervical spine dislocation.The thoracic cavities were opened under sterile condition, the thymus glands situated above the heart were removed and a single cell suspension of thymocytes was produced in RPMI 1640 medium [ICN] supplemented with 10%(v/v) FCS, 100u/ml benzyl penicillin, 100µg/ml streptomycin, and 2mM L-glutamine. Cellularities were determined with a Coulter counter [ZM, Coulter Electronics, U.K.] and adjusted to 10^7cells/ml.

Mitogen: Concanavalin-A (ConA) will itself stimulate thymocyte proliferation. A suboptimal concentration of ConA which will not itself stimulate thymocyte proliferation was determined (1µg/ml) for use during the assay of IL1 activity.

Tritiated thymidine: [methyl-^{3}H]thymidine ([^{3}H]TdR) [Amersham] specific activity, 185GBq/mmol, was diluted to a working solution of 300 KBq/ml with RPMI 1640 medium containing 40IU/ml gentamicin.

100 µl of 10^7 thymocytes/ml and 50 µl of 4 µg/ml ConA were added to a number of flat-bottomed wells of a 96 well microtitre plate (Nunc, Denmark) 50 µl of either conditioned medium, or a range (2 - 100 U/ml) of recombinant human IL1α [Immunex, U.S.A.] to produce an IL1 titration curve, was added to each well. The plate was incubated for 3 days in a 37°C 5% CO_2 in air, fully humidified incubator after which 25 µl of 300 KBq/ml [^{3}H]-TdR was added to each well. Incubation was continued for 16 hours. Cells were harvested using a Titertek cell harvester [Skatron, Norway] onto glassfiber filter paper [Titertek]. After thorough oven drying, individual filter discs were placed in scintillation vials and 2 mls of scintillant [Optiphase 'Safe', FSA Laboratories, U.K.] added. [^{3}H]-TdR uptake by proliferating cells was determined using a liquid scintillation counter[LKB1214, Rackbeta, U.K.]. Each vial was counted for a 5-minute period and a 'count per minute' (CPM) value determined. This figure reflects the proliferation of thymocytes stimulated by IL1.

2-15 [^{3}H] thymidine incorporation assay determining the growth-stimulating activity of sff conditioned medium

2-15-1 Aim and principle

Aim: Demonstration that the proliferation of cells grown in chemically defined medium is enhanced by serum free medium conditioned by HUC-1N cells.

Principle: Measuring the radioactive thymidine incorporation can be used as an index relating to cell proliferation.

2-15-2 Materials and methods

Serum free conditioned medium: SV-HUC-1N cells were grown to confluence in a 80 cm^2 flask, 10 mls of fresh Ham's F12 medium (no serum supplement) was added and incubation was continued as usual. The medium was removed after 3 days and centrifuged at 3000 rpm for 15 minutes then stored at -20°C until required. [^{3}H]TdR [Amersham] was diluted to a working solution of 300 KBq/ml with RPMI 1640 or Ham's F12 medium.

Cells were harvested as previously described [1-2-1] and rinsed once with PBS before resuspension in serum free culture medium. $1x10^5$ cells/well were seeded in a 24 well flat bottom tissue culture plate [Nunc]. Conditioned medium was serially diluted with fresh medium (no serum supplement) and added to appropriate wells. Control wells contained fresh medium only. Plates were incubated in a 5% CO_2 in air, fully humidified incubator at 37°C for 48 hours (HUC-1 cells had one medium change after 24 hours). [^{3}H]TdR was added at 60 KBq/well and incubated for another 8 hours for T24 cells and 16 hours for HUC-1 cells. Medium containing [^{3}H]TdR was removed and plates rinsed twice with warm PBS to remove excess [^{3}H]TdR. Between successive rinses, plates were kept flooded with fluid to protect cells from surface tension effects which may dislodge them from the plastic.(Finlay, G. J. 1984). 1 ml of cold 5%(w/v) trichloroacetic acid (TCA) [BDH] was added to each well and the plates kept at 4°C

for 30 minutes to fix the cells. Plates were rinsed 4 times with 5% TCA and 1 ml of a mixture of 0.1N NaOH and 0.1%(w/v) sodium dodecyl sulphate (SDS) was added to each well and incubated at 37°C for 1 hour to dissolve the acid-insoluble precipitate. After one hour 0.1 ml of 1M HCl solution was added to each well and the total well contents were aliquoted into a scintillation tube containing 10 ml of Optiphase 'safe' [LKB], vortexed and the amount of [^{3}H]TdR incorporated into the proliferating cells determined with a liquid scintillation counter [LKB 1214, Rackbeta]. Results were expressed as a concentration of sff conditioned medium versus [^{3}H]TdR uptake dose response curve.

2-16 Growth inhibitory effects of Roussin's Black Salt (RBS) on exponentially growing cells.

2-16-1 Aim and principle

Aim: Determination of cytotoxic effects induced by RBS

Principle: Cytotoxic effects were calculated form the ratio of living drug treated cells to living nondrug treated cells which were exponentially growing.

2-16-2 Materials and methods

Cell lines: Human urothelial cells, non-tumorigenic SV-HUC-1 (doubling time 40 hours), tumorigenic T24 (doubling time 19 hours).

Drug: Hepanitrosyl-tri-μ3-thioxotetraferrate (mw:553) also known as "Roussin's black salt" (RBS) (Roussin, F. Z. 1858) was synthesized and supplied by Dr. A. R. Butler (Department of Chemistry, University of St. Andrews) in powder form. RBS was diluted serially

in PBS. Drugs solutions were sterilized by filtration through a 0.22 μm pore diameter filter [Millipore].

All these assay were performed in a darkened room and a lamp (40W) filtered with ILFORD 904 safe light glass was used as an illumination source.

A desired number of viable cells ($1x10^5$ cells/flask for T24 cells and $1.5x10^5$ cells/flask for SV-HUC-1 cells) were seeded in 25 cm^2 flasks and incubated under standard condition for 48 hours. Cells were allowed to attach and achieve exponential growth. The supernant medium was removed and flasks rinsed twice with PBS then 4.5 mls of serum free medium added. The whole flask (except cap) was covered with tin foil to exclude light. 0.5 ml of 10x concentrated drug solution was added (0.5 ml of PBS for control group) to begin the assay. After a 60-minute incubation at 37°C, medium containing drug and the tin foil were removed. Flasks were rinsed twice with PBS and 5 mls of fresh serum supplemented culture medium added. Incubation was continued for a period of at least 2 cell doubling times (2 days for T24 cells, 4 days for SV-HUC-1 cells). During this period the cells in control group (PBS-treated) were in exponential growth.

2-16-3 Assessment of the viable cells

Medium was removed and flasks rinsed with trypsin once then 1 ml of fresh trypsin was added. Cells were dislodged after a short incubation period, disaggregated by gently pipetting and diluted in serum supplemented medium. The total volume of cell suspension present was important to allow the calculation of the total number of viable cells. 50 μl of 2%(w/v) fast green [Sigma] in 0.15M NaCl were added to each ml of cell suspension and incubated at 37°C for 10 minutes. Viable cells ,which did not stain green, were counted

with a hemocytometer. Result were expressed as the percentage of cell proliferation, defined by the ratio of viable cells of drug treated cells to non drug treated cells.

2-17 Growth inhibitory effects of laser-irradiated RBS on exponentially growing cells

2-17-1 Aim and principle

Aim: Demonstration the photosensitivity of RBS.

Principle: As [2-16-1].

2-17-2 Materials and methods

Laser irradiations were conducted in the laboratory of Dr. F.W. Flitney's which was darkened and used a red safelight (60W) as the sole means of illumination An Argon ion laser (Spectra Physics Ltd., type 168-09) was the source of irradiated light and 457.9nm wave length light used.

The method used here was the same as [2-16-2] but PBS instead of serum free medium was used as culture fluid for the period of drug exposure and a selected intensity of laser light was used to irradiate the cells. The control group received only PBS. For some experiments RBS was diluted in a 10%(w/v) bovine serum albumin [Sigma] in PBS solution which was sterilized by filtration through a 0.22 μm pore diameter filter [Millipore]. Cellularity of viable cells was determined as [2-16-3].

2-18 Hemoglobin suppression of the growth inhibitory effects of RBS

2-18-1 Materials and methods

Ferro-haemoglobin was prepared by dissolving 1 mM of bovine hemoglobin (Sigma) in PBS. A ten fold molar excess of sodium dithionite ($Na_2S_2O_4$) was added and dialysed against 100 volumes of PBS for 12 hours at 4°C with 3 changes of dialysis fluid to remove the $Na_2S_2O_4$. Ferro-haemoglobin solution was sterilized by filtration through a 0.22 μm pore diameter filter [Millipore]. The concentration of hemoglobin was determined by a hemoglobinmeter (HemoCue, Sweden) and stored in aliquots at -20°C (Martin, W. *et al* 1986). The method was as [2-16-3] and [2-17-2] with the addition of ferro-haemoglobin solution and the cellularity of viable cells was determined as [2-16-3].

2-19 Colony formation survival curve of CHO and xrs cells

2-19-1 Aim and principle

Aim: Demonstration of the possibility that RBS causes DNA damage.

Principle: Using the different survival of two cell lines which differ in DNA repair to indicate DNA damage.

2-19-2 Materials and methods

Cells ($2x10^5$ of CHO, $4x10^5$ of xrs) were grown in 25 cm^2 flasks and kept in a standard culture condition for 48 hours allowed to attach and grown in exponential phase. Medium was removed and flasks were rinsed twice with PBS. Different concentrations of RBS in PBS were added (as a control PBS only was added) and flasks kept at

37°C for 1 hour. Flasks were rinsed twice with PBS and cells detached with TE. Cell numbers were obtained using a hemocytometer. An appropriate number of cells (200-10000) were plated in 90mm tissue culture grade petri dishes with 10 ml of 10%(v/v) FCS Eagle's minimal essential medium and kept in a 5% CO_2 in air, 37°C fully humidified incubator. After 14 days the medium was poured out and the dishes were rinsed with PBS. Cells were fixed with methanol for 10 minutes and stain with 10% (v/v) Giemsa [BDH] in water.for 10 minutes and air-dried.

Scoring:

For each survival curve, triplicate dishes were seeded *per* treatment. Colonies containing more than 50 cells were scored as considered to originate from viable cells Between 20 and 450 colonies were counted for each treatment. The percentage of survival was calculated by reference to controls.

2-20 Micronuclei assay of CHO and xrs cells after exposure to RBS

2-20-1 Aim and principle

Aim: Demonstration of the possibility that RBS causes DNA damage.

Principle: Using the difference in micronucleus formation between two cell lines which differ in DNA repair to indicate DNA damage.

2-20-2 Materials and methods

Cells ($4x10^4$ of CHO and $6x10^4$ of xrs) were grown in a 24 well flat bottom tissue culture plate [Nunc, Denmark] and kept in a 5% CO_2 in air, 37°C, humidified incubator for 48 hours, allowed to attach and grow exponentially. The plate was rinsed twice with PBS before

1 ml of 10 μM of RBS in PBS was added to each well (control cells received PBS only) and plate kept at room temperature for 40 minutes. RBS was removed and the plate rinsed twice with PBS. 1 ml of 10% FCS Eagle's minimal essential medium were added to each well then cytochalasin B (Cyto B; stock solution 3 mg/ml in DMSO) [Sigma] was added to give a final concentration of 3 μg/ml. The plate was kept in a 5% CO_2 in air, 37°C, humidified incubator for 16-24 hours. Cells were detached with TE and dispersed onto clean slides by centrifugation using a Shandon Cytospin 2 (800 rpm for 10 min). Slides were fixed in methanol for 10 minutes, stained in 56% (v/v) Jenner [BDH] in water for 5 minutes and 25% (v/v) Giemsa in water for 10 minutes, with rising in water after each stain.

Scoring:

Binuclear cells containing micronuclei clearly separated from and smaller than one-tenth of the main nucleus, were scored (Arlett, C. F. *et al* 1989). A total of 100 binucleate cells were scored *per* sample *per* experiment. Results were expressed as the total number of micronuclei *per* 100 binuclear cells.

2-21 Turnbull blue reaction of RBS-treated cells

2-21-1 Aim and principle

Aim: Demonstration that RBS is bound to target cells.

Principle: Ferrous salts become blue whilst undergoing the Turnbull reaction (Culling, C. F. A. 1957).

2-21-2 Materials and Methods

One drop of a single cell suspension was pipetted onto one end of a sterile glass slide. Preparations were kept in a humid chamber and incubated in a 37°C, 5% CO_2, humidified incubator overnight. Slides were rinsed 3 times with PBS. RBS was then added to cells which were then kept in a humid chamber for 10 minutes. After rinsing 3 times with PBS to remove unbound RBS, slides were dipped into 5% (w/v) ammonium sulphide (Sigma) for 1.5 hours then rinsed with distiled water. Equal parts of freshly prepared 2% (w/v) potassium ferricyanide and 2% (v/v) hydrocholric acid were mixed together before slides were immersed in the solution and kept there for 30 minutes. Slides were washed thoroughly in distiled water and allowed to air dry.

Chapter III

Tumorigenic transformation of human urothelial cells and characterization of their properties

Summary

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3-1-2-3 Vital stain of cellular foci

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3-1-2-5 Tumor tissue explant culture and development of SV-HUC-1T cells

3-1-2-6 Cloning SV-HUC-1TS cells

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3-1-3-2 Changes of phenotype

3-1-3-3 Tumorigenicity assay of SV-HUC-1N cells

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3-1-3-5 Histopathological features of tumors

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3-1-3-7 Tumorigenicity of tumor cells

3-1-4 Discussion

3-2 Identification of the transformed cells

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3-2-2 Procedure

3-2-2-1 Chromosome studies

3-2-2-2 Immunocytochemical staining of tumor cells

and tumor tissue
3-2-2-3 DNA fingerprinting analysis

3-2-3 Results

3-2-3-1 Chromosome studies
3-2-3-2 Immunocytochemical staining
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3-2-4 Discussion

3-3 Molecular changes associated with transformed cells

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3-3-2-2 Measurement of doubling time of HUC-T cells
3-3-2-3 Growth-stimulating activity of sff conditioned medium
3-3-2-4 In vitro murine HPP-CFC assay of stem cell factor-like effect in conditioned medium
3-3-2-5 ELISA assay for human stem cell factor in conditioned medium
3-3-2-6 Murine thymocyte proliferation assay for IL1-like activity in conditioned medium

3-3-3 Results

3-3-3-1 The fluorescent DNA distribution in cell cycle
3-3-3-2 Doubling time of HUC-T cells
3-3-3-3 Growth-stimulating activity of sff conditioned medium
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3-3-4 Discussion

Conclusion

Fig 3-1-1 Photomicrographs of cellular foci.

Fig 3-1-2 Photomicrographs of vital stained cells within foci.

Fig 3-1-3 Photomicrographs of confluent living cell cultures.

Fig 3-1-4 Photomicrograph of primary culture of tumor tissue.

Fig 3-1-5 Photomicrographs of H&E stained sections of tumors.

Fig 3-2-1 Photomicrographs of model karyotype of cells.

Fig 3-2-2 Photomicrographs of SV40 T antigen stained tumor tissue.

Fig 3-2-3 Photograph of DNA fingerprinting of three types of cells

Fig 3-3-1 Schematic diagram of HPP-CFC assay.

Fig 3-3-2 Schematic diagram of thymocyte proliferation assay.

Fig 3-3-3 DNA histogram of flow cytometry assay.

Fig 3-3-4 Growth curve of SV-HUC-1T cells

Fig 3-3-5 Growth-stimulating activity of sff conditioned medium.

Fig 3-3-6 HPP-CFC assay of stem cell factor-like activity.

Fig 3-3-7 Concentration of h SCF of conditioned medium.

Fig 3-3-8 IL1-like activity of conditioned medium.

Table 3-1-1 Tumorigenicity of SV-HUC-1N cells

Table 3-1-2 Tumorigenicity of SV-HUC-1 cells

Table 3-1-3 Tumorigenicity of SV-HUC-1T cells

Table 3-2-1 Distribution of chromosome numbers of HUC cells

Summary

Tumorigenesis of human urothelial cells is a multistep process. Initially normal human urothelial cells are immortalized by transfection with SV40. Subsequently they become tumorigenic by either exposure to chemical carcinogens or by transfection with a second oncongene (Bookland, E. A. *et al* 1992; Kao, C. *et al* 1992). The objective of this study was to demonstrate that SV40-immortalized human urothelial cells can be tumorigenically transformed by some other means: cells grown in a serum free, factor free, chemically defined culture for a period of time. The tumorigenicity of cells was determined by the generation of a tumor after inoculation into nude mice. The origin of transformed cells was confirmed by comparing the DNA fingerprint profile of both transformant and parental cells. Furthermore a novel band of hypervariable DNA fragment found in transformant cells may indicate mutation. The levels of concentration of human stem cell factor and human IL1 α -like activities were lower in the medium conditioned by transformed cells than by parental cells. This model of transformation may correlate with the heterogeneity of behavior or phenotype of cells within a tumor. It is possible that tumors deprived of normal levels of growth factors and nutrients (eg. around the area of central necrosis) may undergo further transformation thus leading to the heterogeneity in clinical conditions associated with tumor cells.

Outline

In this chapter, tumorigenic transformation of human urothelial cells is discussed in 3 sections.

In the first section, tumorigenic transformation of the cells, a method of transformation is described and the development of tumorigenicity among transformed cells is illustrated.

In the second section, identification of transformed cells, the origin of transformed cells from parental cells is demonstrated and evidence for the possibility of the mutation of transformed cells is presented .

Thirdly changes appearing in transformed cells are reviewed in molecular changes associated with transformed cells.

3-1 Tumorigenic transformation of the cells

3-1-1 Introduction

Properties of transformed cells.

Normal fibroblasts or epithelial cells in primary culture can survive in culture conditions for only a limited period. In the course of cultivation the properties of cells may change, they may undergo alterations and become transformed cells with unlimited life span. The alterations may arise spontaneously or after the treatment of cultures with various agents. Transformed cells differ from normal cells in several ways, the main manifestations including: (1) Development of oncogenicity; cells will form tumors when implanted into syngeneic or immune-deprived mice. (2) Loss of anchorage dependence of growth; cells may grow in suspension or in semisolid media such as agar. (3) Loss of contact inhibition; cells may pile up into colonies or foci. (4) Decreased serum dependence; cells may proliferate in medium with low concentration of serum in which normal cells may be unable to grow. (Vasiliev, J. M. *et al* 1981).

Some DNA-containing viruses such as simian virus 40 (SV40) and polyoma virus have shown the capability to transform fibroblastic and epithelial cultures. Susceptible cells are infected with SV40 in two different ways: "permissive" or "non-permissive". In the course of permissive infection, infectious virions are formed inside the cells. Releasing of mature virus particles causes lysis and cell death. Non-permissive infection is associated with abortive infection and cell survival. A very small portion of abortively infected cells may

carry the viral genome in an integrated state and present with properties of transformed cells. The common feature of SV40-transformed cells is the presence of two proteins which are translated from the early region of viral genome namely large T and small T antigens. Although the mechanism of transformation by SV40 is currently unknown, the possibility of large T antigen playing an important role has been inferred by the evidence that large T antigen is localized in the nucleus of transformed cells. Large T antigen alone is capable of converting normal recipient cells into transformed cells (Kriegler, M. *et al* 1984) and it binds with tumor suppressor gene products (RB and p53) (DeCaprio, J. A. *et al* 1988; McCormick, F. *et al* 1981).

Although several established animal cell culture systems have been used to study the tumorigenesis of epithelial cells, because of species differences and organ specificity, one of the best ways to understand tumorigenesis in the human bladder is to use human urothelial cells to perform *in vitro* studies. The SV-HUC-1 cell line is a normal human urothelial cell line which has been immortalized by SV40, and demonstrates some features of a transformed cell except that it is unable to form tumors in athymic mice (Christian, B. J. *et al* 1987). This cell line has been used for studies of oncogenic agents by assaying their ability to induce tumorigenicity in the SV-HUC-1 cells (Bookland, E. A. *et al* 1992).

Cells grown in culture need an environment which fulfills the requirements for survival and proliferation. To achieve this purpose, serum is the most widely used supplement to an incomplete culture medium. It supplies mainly hormonal factors, attachment and spreading factors and transport proteins (Maurer, H. R. 1986). The composition of sera are complex, not all of them

have been identified and a relatively large variation in components exists between batches and between different species of origin. Thus cells grown in a serum-free culture condition may provide a system which avoids uncertainties imposed by batch variations of undefined serum components (Waymouth, C. 1984). In addition, such a serum-free culture system has advantages for studies of the interaction of hormones or drugs with cells and studies of nutrition and secretion at the cellular and molecular level (Barnes, D. *et al* 1980). Mammalian cells grown in synthetic medium required combinations of factors which can carry out the functions of serum. Occasionally, in the absence of these supplements, a selected autostimulated cell may grow (Hill, M. *et al* 1989). In some cases of serum deprivation simultaneous tumorigenic transformation may occur in primary cell cultures (Andresen, W. F. 1967) and immortal cell lines (Hill, M. *et al* 1991). Because SV40 immortalization of human epithelium does not give rise to a tumorigenic cell (Hill, M. *et al* 1991), we wanted to know whether the SV-HUC-1 cell line will grow in a serum free conditions and furthermore will the cells undergo tumorigenic transformation?

3-1-2 Procedures

3-1-2-1 Isolation of autotrophic variants of SV-HUC-1 cells

SV-HUC-1 cells were grown and passaged as described in [2-1-1]. Different cell densities (from $1.5x10^3/cm^2$ to $3x10^4/cm^2$) were seeded into a serum free, factor free (sff) chemically defined medium for a variable period of time and autotrophic cell variants

which were designated as SV-HUC-1N were isolated as described in [2-3-1].

3-1-2-2 Phenotype changes of SV-HUC-1N cells

Morphological changes of SV-HUC-1N cells were observed and photographed using an inverted microscope equipped with photograph facilities (Diavert inverted microscope [Leitz Wetzlar Germany] equipped with Wild photoautomat), using Agfa 25 film.

3-1-2-3 Vital stain of cellular foci

$1.5x10^5$ of SV-HUC-1TS cells were grown in a 25 cm^2 flask with 7% FCS F12 medium until foci formed and stained with 5μg 5 C-FDA/ml PBS as described in [2-11]. Cells were observed using a laser scanning confocal imaging system which were operated in conjunction with a Nikon diaphot inverted fluorescence microscope [BioRad, MRC 600 USA], 488nm light as a illuminating source.

3-1-2-4 Tumorigenicity assay of SV-HUC-1N cells

Tumorigenicity of cells was determined by assaying the frequency of tumors arising in athymic nude mice after subcutaneous inoculation of 4-10 $x10^6$ SV-HUC-1N cells and tumors were monitored for a period of time as described in [2-4]. SV-HUC-1 cells cultured in 7% or 1% FCS F12 were used in the same protocol as a control studies All the nude mice at the time of starting experiments were 6-8 weeks old. As a positive control, cells of the T24 line, a human bladder carcinoma cell line, which give rise to tumors within 4 weeks were periodically inoculated into the nude mice at a cell dose of $5x10^6$ cells.

3-1-2-5 Tumor tissue explant culture and development of SV-HUC-1T cells

Tumors formed in nude mice were dissected. Small parts of the tumors were examined histopathologically and the rest of tumors were used in tissue explant culture as described in [2-4-3]. Cells grown out from one specific tumor were designated as SV-HUC-1T.

3-1-2-6 Cloning SV-HUC-1TS cells

21 days after $8.8x10^3$ of SV-HUC-1T cells/cm^2 were seeded in semisolid methylcellulose, many colonies formed and a clone of cells from one such colony was designated as SV-HUC-1TS as described in [2-5].

3-1-2-7 Tumorigenicity of SV-HUC-1T and SV-HUC-1TS cells

The tumorigenicity was determined as described in [2-4] and cell doses of 10-15 million cells for each inoculation were used.

3-1-3 Result

3-1-3-1 Isolation of autotrophic cell variants

When SV-HUC-1 cells were transferred to sff conditions, they took more than 24 hours to adhere to the polystrene plastic surface of culture flasks, then cells either remained quiescent or died. After a lag period a few cells started to form colonies and continued to proliferate. These cell variants (SV-HUC-1N) were successfully subcultured and expanded in sff conditions furthermore they could be cryopreserved and rederived in the absence of serum. No evidence of cell survival and proliferation was observed in flasks which were initially seeded with less than $2x10^4/cm^2$ (data not shown).

3-1-3-2 Changes of phenotype

SV-HUC-1N cells piled up to form cellular foci. These foci were observed during exponential growth and post confluence (Fig. 3-1-1) and this phenomenon was not observed in SV-HUC-1 cells. Cells around foci appeared to have a non-random arrangement. They grew in a radiated style with foci at center (not shown). Vital staining of these foci and observation with confocal scanningmicroscope revealed that these foci were composed of living cells (Fig. 3-1-2).

Under the same growth conditions, post confluent cultures of SV-HUC-1T cells became spindle shaped and grew densely with numerous mitoses. While SV-HUC-1 cells still kept their cobble stone appearance with fewer cells in mitosis (Fig. 3-1-3).

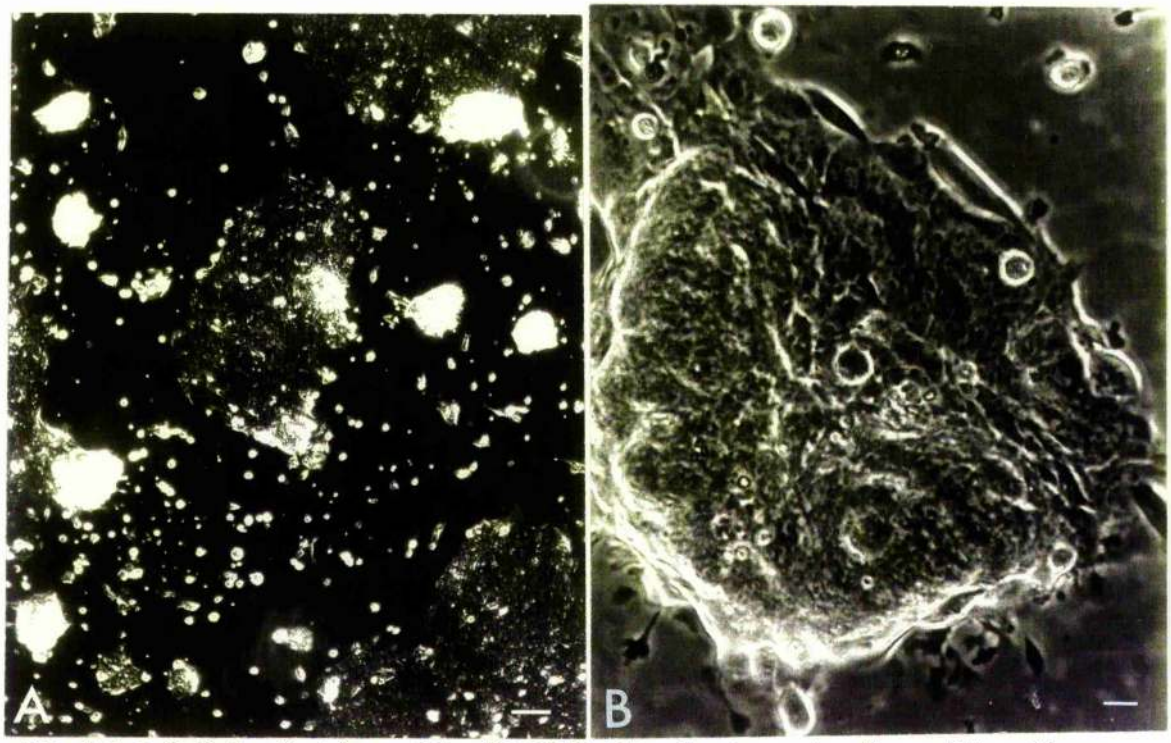


Fig. 3-1-1 Cellular foci formed by SV-HUC-1N cells grown in sff condition. Phase contrast micrograph showing piling up of cells and formation of cellular foci. (A) At low power many foci are seen in the culture flask, each white spot represents one focus of cells. Bar=35μm. (B) The single focus of cells, one cell in mitosis can be seen at the top of cells. Bar=7μm.

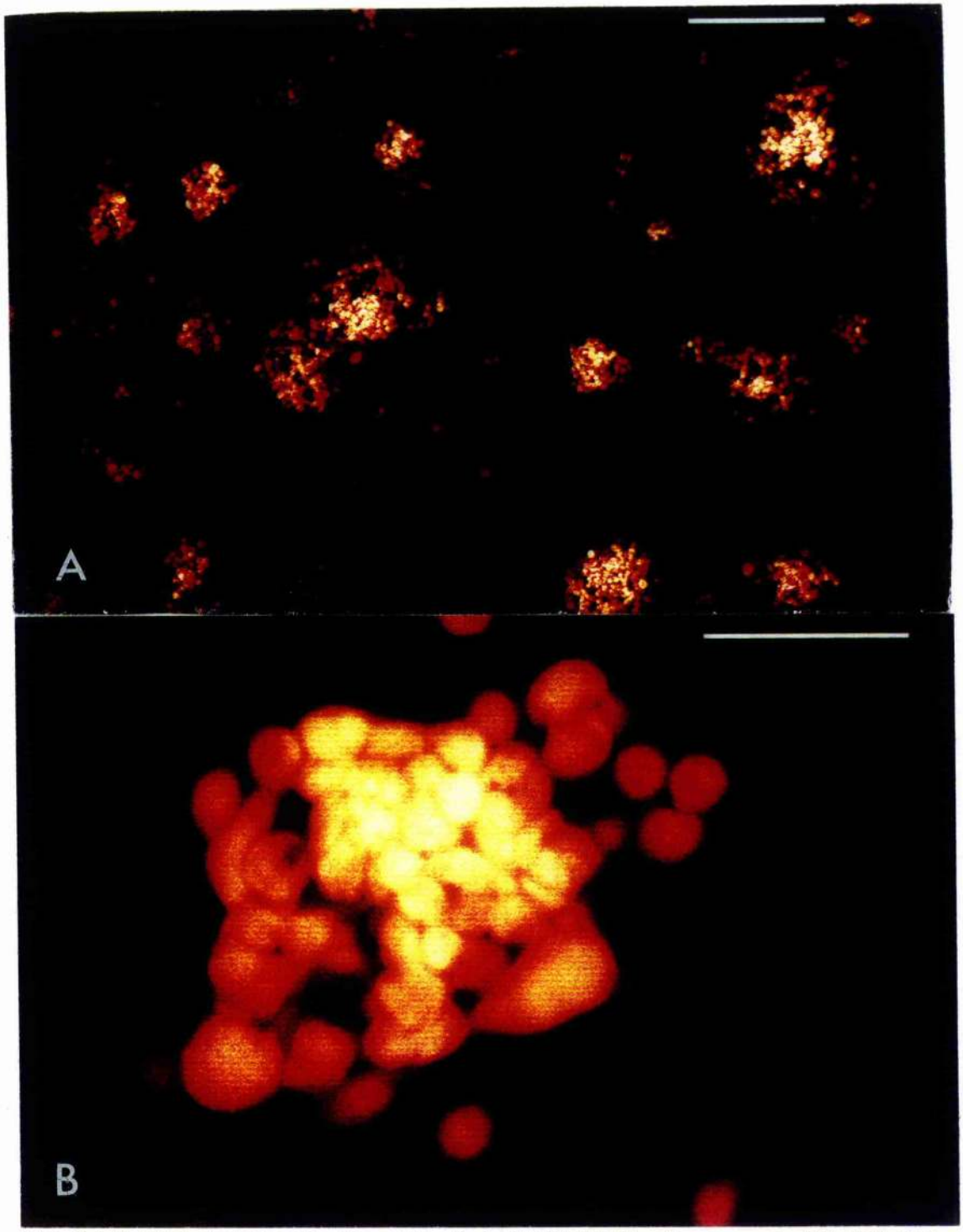


Fig. 3-1-2 Vital staining of cells within foci. SV-HUC-1T cells grown in 7% FCS F12 were stained with 5-carboxyfluoresein diacetate and observed under a confocal laser scanning microscope illuminated with 488nm light. (A) Lower power field showing many cellular foci. (B) Single focus of piling up cells. Cells were artificially orange colored and bright golden staining reflected fluorescent in viable cells. Bars, 100μm for (A) and 50μm for(B).

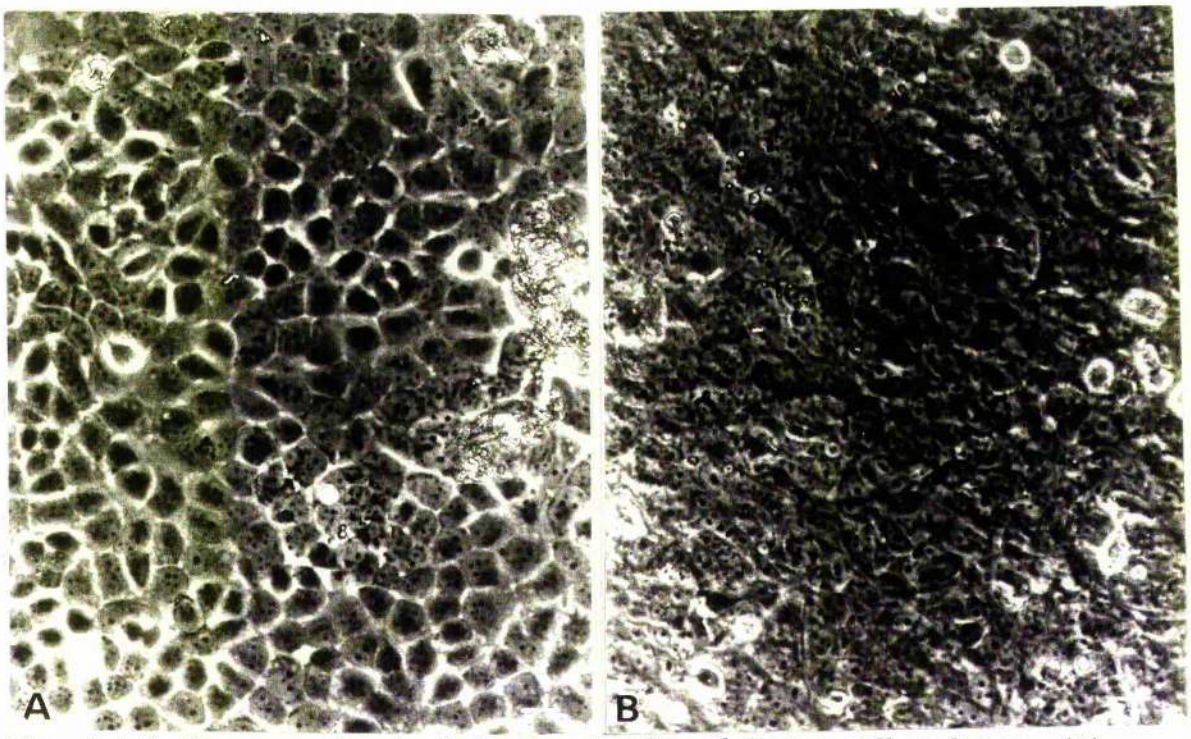


Fig. 3-1-3 Phase contrast photomicrographs of living cell cultures. (A) Postconfluent (30 days in culture without passage) culture of SV-HUC-1 cell, cobble stone appearance of cells with few mitoses. (B) Postconfluent culture of SV-HUC-1T cells showing more densely packed cells with numerous mitoses.Bar=7μm for (A) and (B).

3-1-3-3 Tumogenicity of SV-HUC-1N cells

Subcutaneous inoculation of cells sometimes resulted in the formation of a small soft cyst-like lesion which disappeared within ten days. In this experiment, a tumor was defined as a hard palpable mass which arose and persisted after the initial swelling was gone. At different stages in the course of expansion, SV-HUC-1N cells were inoculated into nude mice (table 3-1-1 column 2). There were seven experimental groups with a total of 17 nude mice. Four mice developed tumors (table 3-1-1 column 3). Tumors grew in nude mice from 13.6 to 32 weeks before being dissected (table 3-1-1 column 4). Almost all tumors reached the size of 3-5

mm in diameter except one in group 3 which progressed to 12 mm in diameter (data not shown). The cell doses for each inoculation were 4-10 million cells (table 3-1-1 column 5).

Group	Weeks in sff medium	Tumor frequency	Weeks * of tumor growth	Cell dose (million)
1	24.9	0/2		7.2
2	24.9	1/3	32	7
3	35.9	1/2	19.6	5
4	34	0/3		4
5	40.5	1/3	13.6	5
6	45	0/2		10
7	44.5	1/2	20	10

* duration between cell inoculation and tumor dissection in weeks.

Table 3-1-1 Tumorigenicity assay of SV-HUC-1N cells

In control studies, continuously passaged SV-HUC-1 cells (table 3-1-2 column 2) and a population of cells adapted to and continuously cultured in 1% FCS F12, were used (table 3-1-2 column 4). No tumor developed in either control experiments (table 3-1-2 column 3) with similar cell doses (table 3-1-2 column 5).

Group	Weeks in medium	Tumor frequency	% FCS in Media	Cell dose (million)
1	27.7 (P56)	0/3	7	10
2	74.28 (P78)	0/2	7	10
3	122.3 (P96)	0/5	7	10
4	25.5	0/2	1	9
5	26.5	0/3	1	13

Table 3-1-2 Tumorigenicity assay of SV-HUC-1 cells

3-1-3-4 Tumor tissue explant culture

The tumor from a mouse in group 3 was used to derive explant cultures in 7% FCS F12 medium. Although at the beginning a mix epithelial and fibroblastic culture was observed (Fig. 3-1-4), after a

few passages fibroblasts gradually disappeared. The remaining epithelial outgrowth of tumor tissue were successfully grown and subcultured and were designated as "SV-HUC-1T".

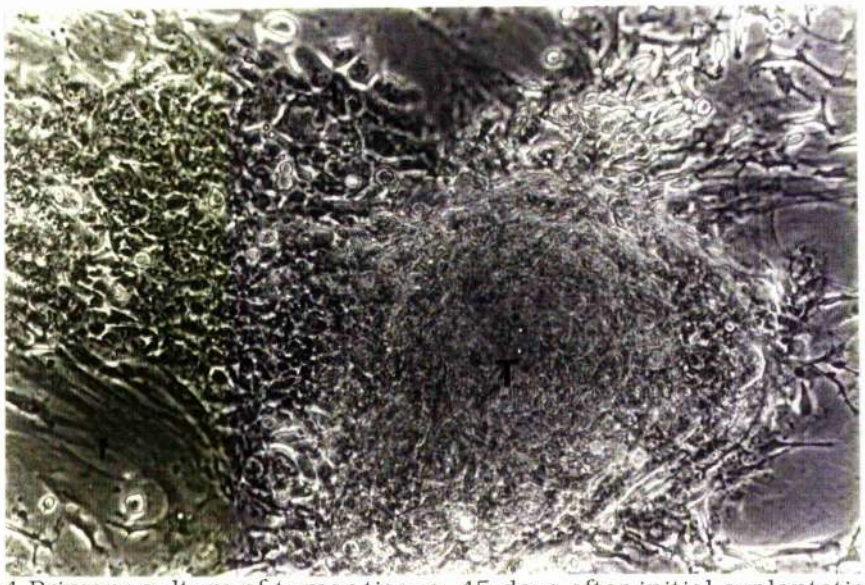


Fig. 3-1-4 Primary culture of tumor tissue. 45 days after initial explantation, tumor cell(t) migrated out from tumor tissue(T) and was surrounded by mouse firoblast(f). Tumor tissue was grown in 7% FCS F12 medium. Bar=14μm.

3-1-3-5 Histopathological features of tumors

Formalin fixed and wax embeded tumor sections were stained with H&E. The picture shown in Fig. 3-1-5 was a typical appearance of tumors, epithelial-like cells were arranged in a papillary pattern with fibrotic central cord. No large variations of nuclei size were observed. The type of cells and the grade of cell differentiation are similar in tumors which were either induced by SV-HUC-1N, SV-HUC-1T or SV-HUC-1TS cells.

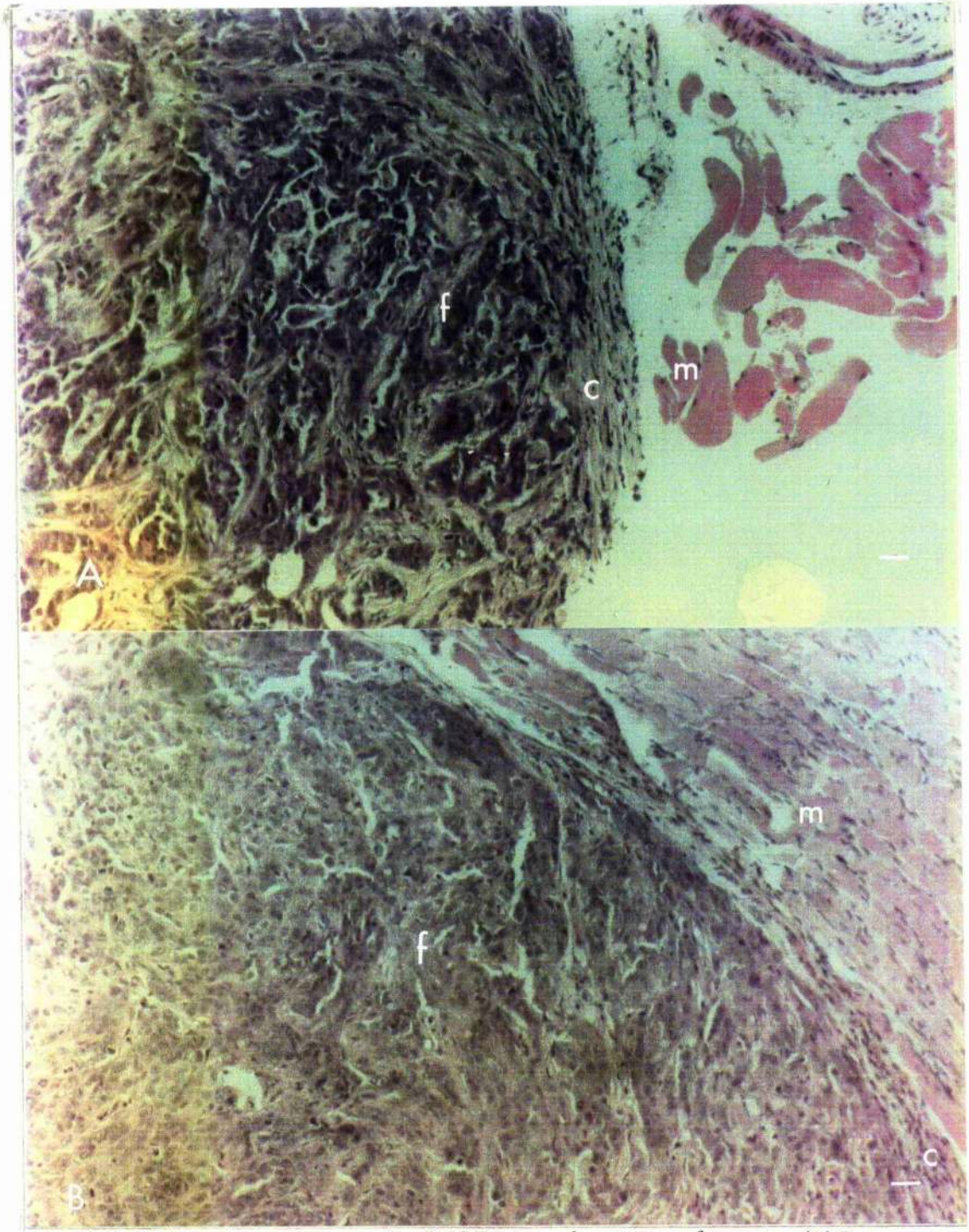


Fig. 3-1-5 Light photomicrograph of H&E stained sections of tumors. (A) Tumor was produced after inoculation of SV-HUC-1N cell, epithelial-like cells are arranged in a papillary pattern with fibrotic central cord(f). Note similar size of round and oval nuclei. A fibrous capsule(c) and muscle(m) surrounds the tumor. (B) A similar histological appearance is present in this tumor which was induced by SV-HUC-T cell. Bar=2.75μm for (A), (B)

3-1-3-6 Clone of tumor cells

Four clones of SV-HUC-1T cells were obtained from colonies which formed in methylcellulose. Only one clone from these colonies was expanded in 7% FCS F12 medium and designated as "SV-HUC-1TS".

3-1-3-7 Tumorigenicity of tumor cells

Using the same protocol, the tumorigenicity of cloned and non-cloned tumor cells was determined. The results were shown in table 3-1-3. Non-cloned tumor cells were inoculated into 8 mice and all of them developed tumors while cloned tumor cells injected into 7 mice only 3 developed tumors. All tumors were within 3-5 mm diameter size range.

Group	Cell type	Tumor frequency	Weeks * of tumor growth	Cell dose (million)
1	HUC-1T	4/4	9.6, 12.4, 15.1, 18.1	15
2	HUC-1T	3/3	12.3, 16.1, 23	12.5
3	HUC-1T	1/1	28	15
4	HUC-1TS	0/2		10
5	HUC-1TS	0/2		10
6	HUC-1TS	3/3	5.5, 11.4 11.4	10

* duration between cell inoculation and tumor dissection in weeks.

Table 3-1-3 Tumorigenicity assay of tumor explantation cells

3-1-4 Discussion

SV40-transformed human cells are rarely tumorigenic in nude mice despite the fact that these cells often exhibit properties commonly ascribed to a neoplastic phenotype, including anchorage independent growth (Chang, S. E. 1986). Expression of SV40-associated function (immortalization) may represent the initial

stage of multistep tumorigenesis of SV-HUC-1 cell (Bookland, E. A. *et al* 1992). Consistent with this model the SV-HUC-1 cells were transformed by depriving them of exogenous growth factors and serum components in the culture environment (sff). A few phenotypic changes were observed, including cells piling up to form foci in the culture flask, higher cell saturation density and most prominently the ability to induce tumors in nude mice.

If the above mentioned transformation is a transient phenomenon then tumor cells in serum supplement medium may reverse this transformation. We have proved that this is not the case by growing sff derived tumorigenic cells in serum supplemented medium, they retained all three properties. Specifically focus formation is not seen as frequently as in sff situation, these cells still have high saturation density and their tumorigenicity persisted.

Cells in culture may exhibit unstable DNA and spontaneous tumorigenic transformation of a human urothelium has been reported (Christensen, B. *et al* 1993). However no tumor was observed when long term (up to two years) continuously cultured parental cells were inoculated periodically into nude mice which may indicate that this tumorigenic transformation is not occurring spontaneously.

When parental cells were cultured in lower serum condition (from 7% to 1%) for long term (6 months), no tumorigenic transformation was observed among these cells [table 3-1-2]. For this reason M. Hill (1991) ruled out the possibility of selection of pre-existent tumorigenic cells. However as R. A. Weinberg (1989) proposed the environment may have an inhibitory effect on the oncogene-bearing cell, a clonal selection model may still be postulated in this experiment. If tumorigenic cells are grown along

with non-tumorigenic cells but form only a small proportion of the whole cell population, the tumorigenic cells will not become dominant because non-tumorigenic cells may exert an inhibitory influence on the growth of the tumorigenic cells. When grown in protein deprived medium non-tumorigenic cells died and inhibitory effects on tumorigenic cells are demolished. Hence tumorigenic cells became dominant in the population and express their tumorigenicity.

Clinical bladder cancers are heterogeneous in their histo-pathological subtype and growth behavior. In vitro, clonally derived SV-HUC cells demonstrated the capability to give rise to different histological phenotypes during tumorigenesis (Bookland, E. A. *et al* 1992). In our experiment, tumorigenicity is markedly different between a clonal tumor cell (SV-HUC-1TS) and its original tumor cells (SV-HUC-1T). This may reflect that diversity.

Other factors may explain why some cells grow better in a sff condition than in a serum supplemented medium. Cells exposed to a factor(s) in serum may compromise the cell's ability to bind and hence respond to another factor(s) (Cross, M. *et al* 1991) As transforming growth factor ß (TGF ß) may play an inhibitory role in the growth of human megakaryocyte, while in sff conditions no such inhibitor(s) existed and cells gained optimal growth (Berthier, R. *et al* 1993).

3-2 Identification of the transformed cells

3-2-1 Introduction

In last section data was presented which demonstrated that SV-HUC-1 cells became tumorigenic after growing in sff condition. However there are two issues which in connection with the origin of tumor cells need to be clarified before we can be convinced that SV-HUC-1 cells were tumorigenically transformed. Firstly we need to ask the question do the tumors that arise in mice develop from the cells which were injected or are they spontaneous events? In other words we need to prove that cells derived from mice tumors or tumor tissues are of human epithelial cell origin.

Microfilaments, microtubules and intermediate size filaments are components of the cytoskeleton present in the cytoplasm of vertebrate cells. Intermediate size filaments containing keratin-like proteins (cytokeratins) are characteristic of epithelial cells. The cell type specificity of the proteins is largely conserved during cell transformation and tumor development (Moll, R. *et al* 1982) can be used to distinguish between epithelial and non-epithelial cells.

SV-HUC-1 cells positively react with antibodies against both SV40 T antigen (Christain, B. J. *et al* 1987) and p53 (personal communication with Dr. M. Armitage). Thus we can also use these properties to determine the origin of cells.

Another issue we must address is to rule out the possibility of cross contamination with another human tumorigenic cell line which could also produce tumors in nude mice. Cross-contamination of cell lines by other cells does not only happen frequently ,but also

occurs in a wide range of cell types. This may go undetected unless unexpected experimental data or obvious morphological differences give reason to suspect the line. There are for example five separate human eosophageal squamous carcinoma cell lines which originate from the same source and a commercially available myocardial line (Girardi cell from Flow Laboratories) which are actually HeLa cells (van Helden, P. D. *et al* 1988). Five human bladder cell lines are cross-contaminated by T24 cells (O'Toole, C. M. *et al* 1983) and HeLa cells are thought to have possibly invaded more than 80 individual lines (Nelson-Rees, W. A. *et al* 1981). The source of errors can not be found easily but the possibilities of an aggressive cell line invading cultures and gradually replacing the cells over a number of passages, or an aggressive line invading flasks in which dormant tumor cells are being maintained and those cells being mistakenly grown as new lines have been proposed (van Helden, P. D. *et al* 1988). In the field of cell biology the production of consistent experimental data in research and the reproducibility required in industrial scale processes depend on the availability of reliable sources of authenticated cultures. There are several methods to identify the origin of cells i.e. chromosome banding, HLA antigen typing, growth characteristics, tumorigenicity, expression of viral antigen (Conner, B. R. *et al* 1980), indistinct histogenesis, isoenzyme electrophoresis (Harris, N. L. *et al* 1981) and immunofluorescence (Nelson-Rees, W. A. *et al* 1981). Two of the most commonly used are cytogenetic and isozyme analysis which can monitor a cell line for chromosome defects and contamination with other cell types. However cytogenetics is a time-consuming method and needs expert interpretation. It may not identify intra-species cross contamination and is not suitable

for cell lines showing cytogenetic variation over short term passage (Miele, M. *et al* 1989). With human cell lines seven or more enzymes are required to provide the identification of an allozyme phenotype with a high degree of confidence (Hay, R. J. 1988) and the probability of chance identity increases when many cell lines of a collection are typed for genetic uniqueness (O'Brien, S. J. *et al* 1980). Multilocus DNA fingerprinting with probes 33.6 and 33.15 is proving to have the ability to both specifically identify a cell line and the ability to recognise contamination by other cell lines (van Helden, P. D. *et al* 1988; Gilbert, D. A. *et al* 1990). Mutation of cell lines may also go undetected for its effects on the characteristics of the cell line may be insignificant in the early stages. DNA fingerprinting has been used to identify mutations in lines cultured at different laboratories of the same origin (Thacker, J. M. *et al* 1988). This approach has also proved successful in the identification of somatic change in cancer (Thein, S. L. *et al* 1987) and the detection of differences in fetal and trophoblast samples (Butler, W. J. *et al* 1988) Alterations in culture supplements and adaptation to serum-free conditions do not appear to have any effect on the fingerprint profile (Stacey, G. N. *et al* 1991). R. Carter (1989) selected a pair of transcribable vectors pSPT 18 and pSPT 19 which contained opposed RNA polymerase promoters from T7 and SP6 phages flanking the pUC18 multiple cloning site, to recombine with minisatellite regions of 33.6 and 33.15 to yield four RNA probes pSPT 18.6, 19.6, 18.15, and 19.15 . The advantages of RNA probes over conventional DNA hybridization probes are: 1. A high and consistently achieved specific activity. 2. A highly defined nature; a single nucleotide chain of defined length, and no competing strand. 3. Ease of preparation. 4. An RNA/DNA

heteroduplex is more stable than a DNA/DNA duplex. It was therefore decided to use pSPT 18.15 RNA probe for use during fingerprinting analysis performed in this project.

3-2-2 Procedure

3-2-2-1 Chromosome studies

At late passages of SV-HUC-1T cells no obvious fibroblast contamination was observed. Cells were prepared for chromosome studies as described in [2-6] using colcemid (0.04μg/ml) to accumulate metaphase cells for 3 to 4 hours. Chromosomes were observed under oil immersion by a Leitz light microscope.

3-2-2-2 Immunocytochemical staining of tumor cells and tumor tissue

$5x10^4$ SV-HUC-1T cells grown on a glass slide or dewaxed tumor tissue, were treated with a mouse monoclonal antibody against human cytokeratins (DAKO-M821), SV40 T antigen(PAb 405), or p53 (DO1). Then amplified with a rabbit anti-mouse IgG, HRP conjugated polyclonal antibody (DAKO-P161). Positive staining was revealed after HRP was reacted with 1mg/ml of DAB plus 0.06% (v/v) of H_2O_2 as described in [2-7]. Each experiment had a duplicate negative control in which no first antibody was added.

3-2-2-3 DNA fingerprinting analysis

About $1.5x10^7$ of SV-HUC-1, SV-HUC-1T, SV-HUC-1TS, and T24 cells were used. Detailed procedures are contained in appendix I. Briefly the genomic DNA was extracted with phenol and alcohol, Hae III was used to digest the DNA, 0.8% (w/v) agarose gel electrophoresis separated the DNA fragments then Southern blotting onto a nylon membrane was performed. Finally fragments

were hybridized with a radiolabelled pSPT 18.15 RNA probe. The autoradiographic results were developed on an x film.

3-2-3 Results

3-2-3-1 Chromosome study

25 metaphase spreads each from SV-HUC-1N and SV-HUC-1T cells were analysed. The distribution of chromosome numbers is shown in table 3-2-1. Both lines showed a modal number of 43, although a few metaphases in the SV-HUC-1N cells contained a fewer number of chromosomes. it is concluded that the chromosomal pictures for the two lines is very similar (Fig. 3-2-1).

Fig. 3-2-1 Photomicrograph of model karyotype of (A) SV-HUC-1T and (B) SV-HUC-1N cells. x 5200.

	Chromosome numbers													
	20	32	35	39	40	41	42	43	44	45	64	71	80	88
SV-HUC-1T		3		1	2	3	2	7	4	1	1			1
SV-HUC-1N	1	1	1	2	3	2	2	6	4			1	1	

Table 3-2-1 Distribution of chromosome numbers of 25 metaphase spreads from SV-HUC-1T and SV-HUC-1N cells.

3-2-3-2 Immunohistochemical staining

Both SV-HUC-1 and SV-HUC-1T cells were positively stained with p53 and human cytokeratin immunohistochemical staining. Positive SV40 T antigen staining was revealed on the wax embedded tumor tissue as shown in Fig. 3-2-2.

3-2-3-3 DNA fingerprinting analysis

The autoradiograph of a DNA fingerprint was shown in Fig. 3-2-3. Lane 1 to lane 3 represent the hybridizing DNA fragments of SV-HUC-1, SV-HUC1T and T24 cells respectively. The cellular genomic DNA was digested with Hae III and hybridized with pSPT 18.15 RNA probe. About 16 resolvable hypervariable fragments are observed in each lane in the 4-20 kilobase size range. Lane 1 and lane 2 shared all except one (arrow) band and no band sharing was noted in DNA fragments bigger than 5.6 Kb between lane 3 and lane 2 (or lane 1). This is supposedly because cells of lane 1 and lane 2 are from the same origin, different from the cells of lane 3.

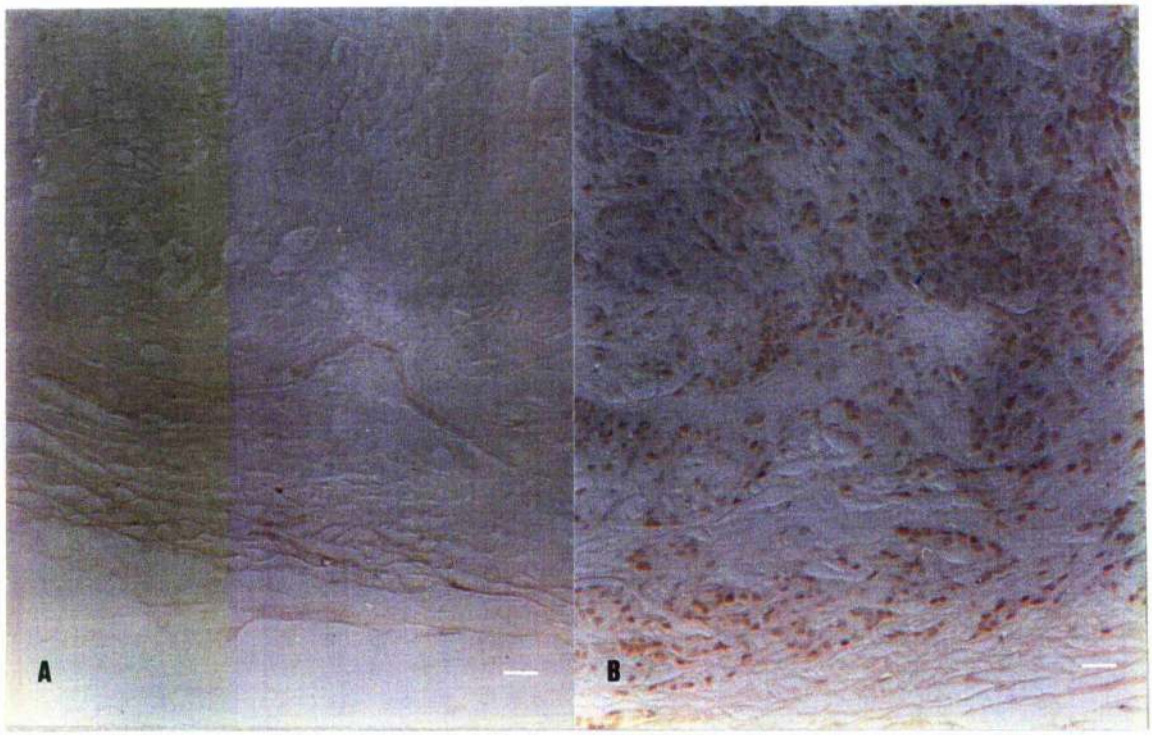


Fig. 3-2-2 Immunohistochemical staining of SV40 T antigen of wax embeded tumor tissue from a nude mouse. (A) Negative control, without primary antibody (B) Stained tissue, brown deposits represent the sites of T antigen.Tumor was produced after inoculation of the SV-HUC-1N cells. Specimen was dewaxed, reacted with antibodies and developed with 1mg/ml of DAB at the presence of 0.06% H2O2 and 0.03% nickel sulphate. Primary antibody was PAb405, a mouse monoclonal antibody against SV40 T antigen. Secondary antibody was DAKO-P161, a enzyme linked rabbit polyclonal antibody against mouse IgG.

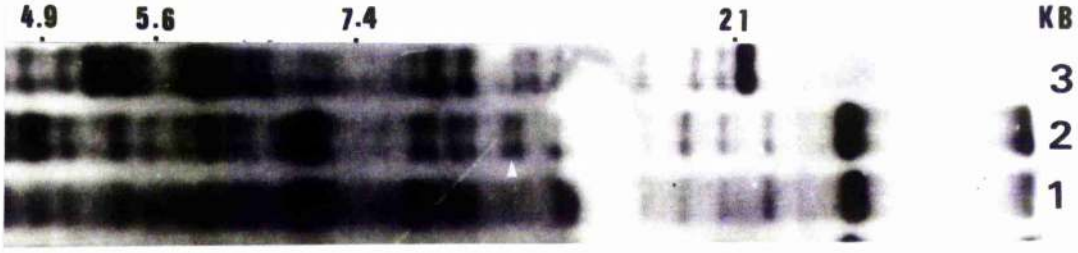


Fig. 3-2-3 DNA fingerprinting of three cell lines. Cellular genomic DNA was extracted, digested with Hae III and hybridized with pSPT 18.15 RNA probe. number on the right side of each lane indicates different cell types 1. SV-HUC-1, 2. SV-HUC-1T, 3. T24. Arrow indicates the site where novel band appears in SV-HUC-1T cells.

3-2-4 Discussion

SV-HUC-1T cells, derived from a tumor which were produced by human urothelial cells and grown in nude mice, show a similar chromosome picture to that of the parental cells. Immunohistochemical staining of this cell confirmed that it contained characteristics of human epithelium cells and had been transfected with SV40 T antigen. All these indirectly support the idea of SV-HUC-1T cells being a tumorigenic transformant of SV-HUC-1 cells. Direct evidence of cell origin comes from the DNA fingerprinting analysis. In Fig. 3-2-3, lane 1 and lane 2 shared 15 resolvable fragments. A mean probability of band sharing of 0.2 was estimated by A. J. Jeffreys (1985b). The chances that two cell line with different origin shared 15 hypervariable DNA fragments are very low (0.2^{15}=~$3x10^{-11}$). This means that cells of lane 1 (SV-HUC-1) and cells of lane 2 (SV-HUC-1T) have the same origin. In a survey of 27 individuals and their parents, one band in 240 clearly resolved offspring bands could not be traced to one or other parent. This gives a mutation rate to a new allele for these hypervariable fragments of 0.004 (1/240) (Jeffreys, A. J. *et al* 1985a). There are at least 10 bands discrepancy between lane 3 and lane 2 (or lane 1 as well). If cells of lane 3 (T24) and cells of lane 2 come from the same origin, then all these 10 bands difference must be a result of mutation. The probability that this is correct is very low (0.004^{10}=~10^{-24}). These two pieces of evidence taken together may indicate that SV-HUC-1T cells are derived from SV-HUC-1 cell and not simply contaminated by T24 cells.

In a DNA fingerprint analysis of 35 patients, changes were observed in cancer cells, including alterations in the relative intensities of

hybridizing DNA fragments in 10 cases and, in 3 cases, the appearance of novel minisatellite fragments not seen in the corresponding individual normal leukocyte DNA profiles (Thein, S. L. *et al* 1987). This appearance of a novel fragment in a DNA fingerprinting profile of diseased cells was also found in a Proteus syndrome affected monozygotic twin brother(Schwartz, C. E. *et al* 1991). The same observation is detected in our result, a novel band appears in the DNA fragments of SV-HUC-1T cells [arrow in Fig. 3-2-3]. Although this change could be due to a spontaneous mutation, as mentioned, the probability is low. It is possible that this mutation is caused by growing SV-HUC-1 cells in sff conditions and as a result the cells are tumorigenically transformed.

3-3 Molecular changes associated with transformed cells

3-3-1 Introduction

The evidence from section 2 has shown that a tumorigenic cell line was derived from a non-tomorigenic cell line. In other words two cell lines shared the same genomic background with different phenotypic characteristics. Further studies should focus on changes occurring during the process of tumorigenic transformation. Firstly, does sff conditioned medium contain factors which support cells grown in sff situations? Soluble factors, including growth factors, which play a major role in controlling ordered cell growth and differentiation *in vivo*, might also have a role in tumorigenic transformation as virally transformed cells displayed lower requirements for exogenous sources of growth factors than did normal cells (Buick, R. N. *et al* 1992). Is there any change in levels of growth factors in the medium conditioned by tumorigenic cells and non-tumorigenic cells?

3-3-2 Procedure

3-3-2-1 Flow cytometry measuring the fluorescent DNA distribution in cell cycle

4μg/ml of propidium iodide (PI) was used to stain the DNA of alcohol fixed SV-HUC-1 and SV-HUC-1T cells and measured with a flow cytometer as described in [2-9]. Data for the measurements of forward scatter versus fluorescence of PI were accumulated in the

FACScan and analysed using a software package "Consort *30" and "DNA" software.

3-3-2-2 Measurement of doubling time of HUC-T cells

$1x10^5$ of SV-HUC-1T cells were plated in $25cm^2$ flask, cultured with 7% FCS F12 medium. Cell number and viability were determined at a regular intervals as described in [2-10] and data used to plot growth curves.

3-3-2-3 Growth-stimulating activity of sff conditioned medium

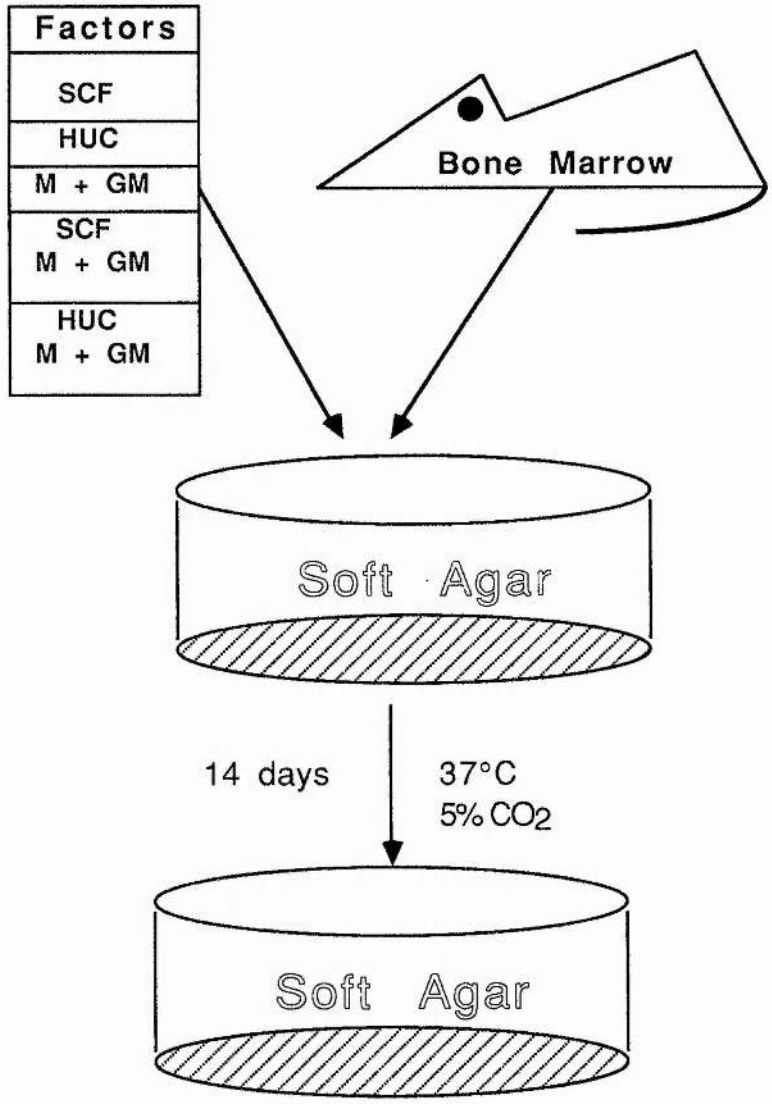
This assay was described in [2-15]. F12 medium conditioned by SV-HUC-1N cells under sff situation was used. $1x10^5$ T24 or SV-HUC-1 cells were seeded in each well of a 24 well flat bottom tissue culture plate containing 2 ml of serially diluted (50% to 3.1%) sff conditioned medium. Serum free medium (RPMI for T24 and F12 for HUC-1 cells) were used as diluent and wells containing no cell as a back ground control. Cultures were incubated for 48 hours then 60KBq of [^{3}H]TdR added to each well and incubation continued for another 16 hours for HUC-1 cells and another 8 hours for T24 cells. Data of [^{3}H]TdR uptake of 3 wells for each treatment were collected and the growth-stimulating activity was expressed as a ratio of averaged [^{3}H]TdR uptake by conditioned medium treated cells over that of concurrently processed control cells. A dose response curve of growth-stimulating effect of increased conditioned medium was plotted.

3-3-2-4 In vitro murine HPP-CFC assay of stem cell factor-like effect in conditioned medium

7% FCS F12 medium conditioned by SV-HUC-1 and sff F12 medium conditioned by SV-HUC-1N cells was used alone or in combination with optimal concentration of rmu GM-CSF (40 U/ml), optimal

concentration of rhu M-CSF (50 U/ml) (personal communication with Dr S. N. Robinson) as the schematic diagram show in Fig. 3-3-1. rmu SCF was used as positive control of synergistic factor at 10 ng/ml. $4x10^4$ murine bone marrow cells were seeded in each semisolid agar dish and the synergistic activity of the conditioned media was assayed as described in [2-12].

Fig. 3-3-1 Stem cell-like activity assay



Colony forming assay

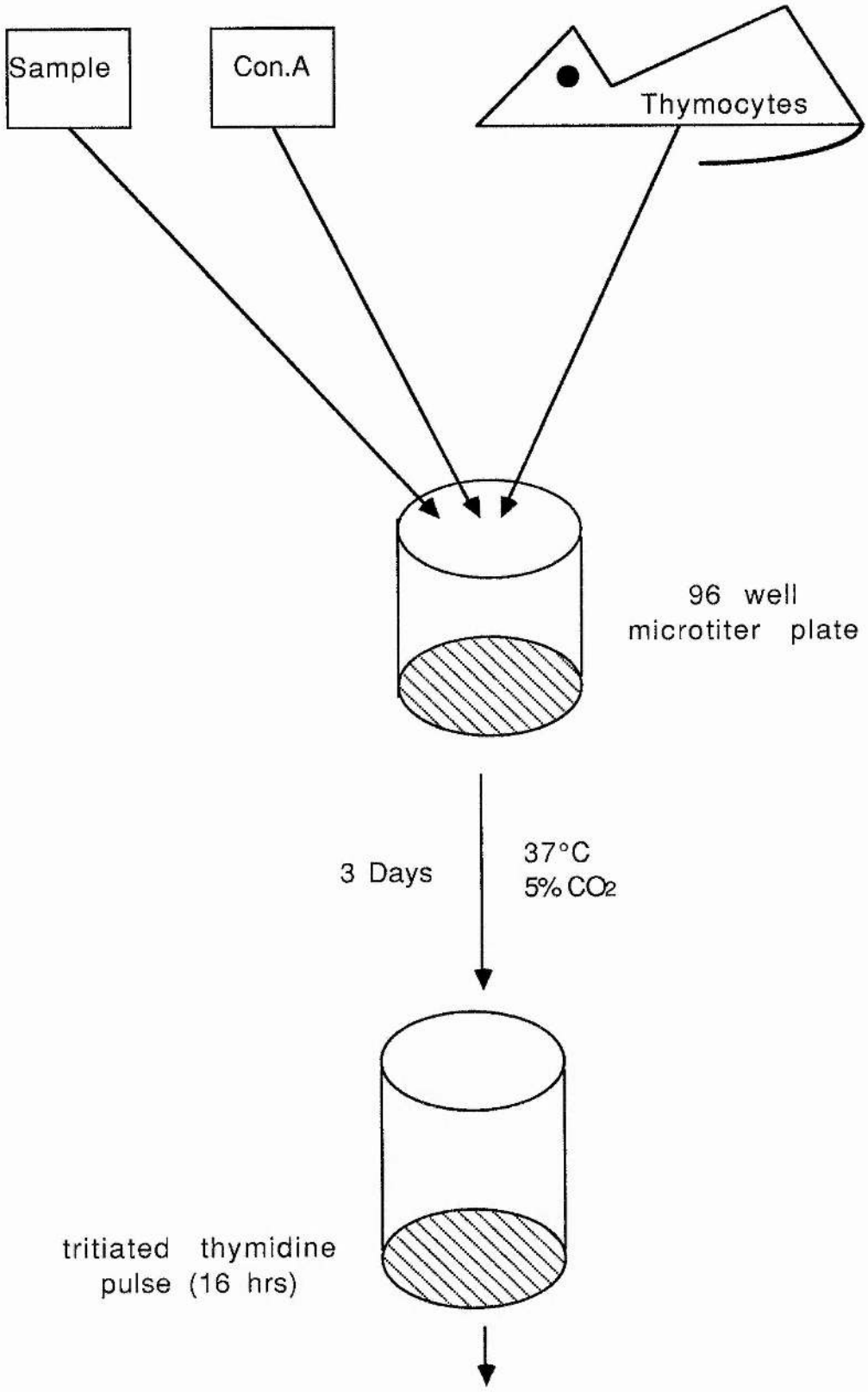
3-3-2-5 ELISA assay for human stem cell factor in conditioned medium

7% FCS F12 medium conditioned by SV-HUC-1 and SV-HUC-1T cells were assayed using BIOTRAK and Quantikine ELISA kits [2-13] procedure as *per* manufactures instructions. Ham's F12 medium was used as diluent, Plates were read with a Dynatech 5000 spectrophotometer at 450 nm. Plain F12 medium containing 7% (v/v) fetal calf serum as a negative control.

3-3-2-6 Murine thymocyte proliferation assay for IL1-like activity in conditioned medium

7% FCS F12 medium conditioned by SV-HUC-1 and SV-HUC-1T cells were assayed. Thymocytes were harvested from 2 to 3 weeks old CBA/H mice as described in [2-4]. A suboptimal concentration of ConA of 1μg/ml was used in this assay (personal communication with Dr S. N. Robinson). To generate a standard IL1 titration curve 100 μl of medium containing $1x10^6$ thymocytes, 50 μl of ConA (4μg/ml) and 50 μl of a range (2 to 100 U/ml) of recombinant human IL1 α were added to made up a total 200 μl in each well of a 96 well microtitre plate. To assay the IL1-like activity in the conditioned medium, 50μl of conditioned medium was added to the wells instead of IL1-α. After 3 days culture, 25μl of 300 KBq/ml [^{3}H]-TdR was added to each well and incubated for 16 hours. Cells were harvested onto a glassfiber filter paper and radioisotope uptake by proliferating cells was determined using a Rackbeta liquid scintillation counter.A simplified schematic diagram of the procedure was shown in Fig. 3-3-2 and described detailly in [2-14]. The IL1-like activity in conditioned medium was expressed in U/ml as read from the equivalent level of uptake in the IL1-α standard titration curve.

Fig. 3-3-2 Thymocyte Proliferation Assay



3-3-3 Results

3-3-3-1 The fluorescent DNA distribution in cell cycle

DNA histogram of tumorigenic (HUC-1T) and non-tumorigenic (HUC-1) cells were shown in Fig. 3-3-3. G0/G1 peak were at 61 and 58.9 channel respectively. Modified DNA index (DI) is a value given to express the amount of DNA content ratio and is calculated by the following equation.

$$DI = \frac{\text{modal channel NO. of G0/G1 peak of tumorigenic cells}}{\text{modal channel NO. of G0/G1 peak of non-tumorigenic cells}}$$

No abnormal peaks of fluorescence intensity were observed in tumorigenic cells in comparison with non-tumorigenic cells.

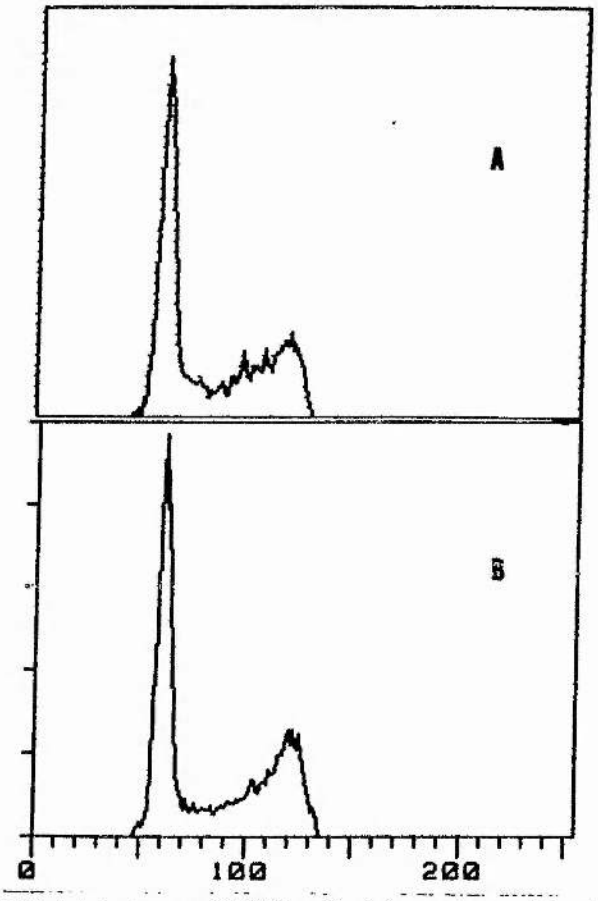


Fig. 3-3-3 DNA histogram of HUC cells (a) nontumorigenic cell SV-HUC-1, G0/G1 peak at channel 58.9. (b) tumorigenic cells SV-HUC-1T, G0/G1 peak at channel 61.

3-3-3-2 Doubling time of HUC-1T cells

The log phase growth curve of HUC-1T cells was shown in Fig. 3-3-4 each point had at least 3 independent samples. The doubling time was measured as 40 hours which was the same as its parent HUC-1 cells.

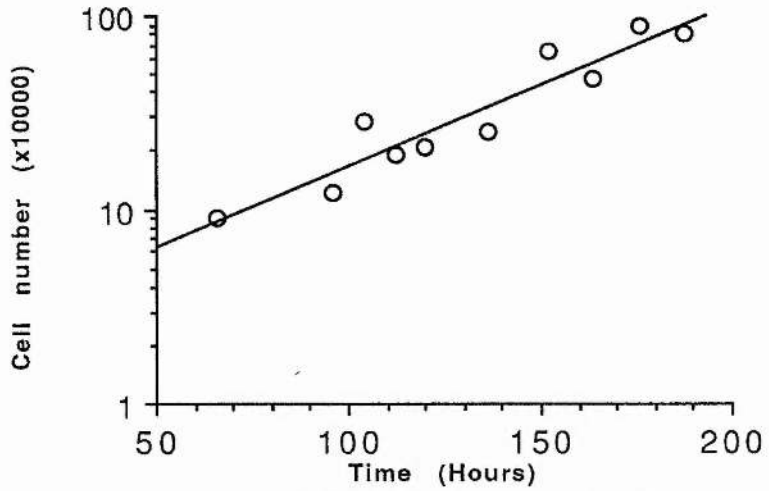


Fig. 3-3-4. Growth curve of SV-HUC1T cells in log phase culture in 7% FCS F12 medium.

3-3-3-3 Growth-stimulating activity of sff conditioned medium

The result of full range of serially diluted conditioned medium using SV-HUC-1 as target cells was shown in Fig. 3-3-5a. Optimal growth-stimulating effect were observed at 12.5% (v/v) of conditioned medium, further increased concentration of sff conditioned medium had less growth stimulation effect. A similar pattern of dose response effect was observed with T24 and HUC-1 cells as indicator cell in a range of optimal concentration of conditioned medium as shown in Fig. 3-3-5b and each data point represented three independent samples. The sff conditioned

medium demonstrated a non-cell specific growth-stimulating effect in SV-HUC-1 and T24 cells.

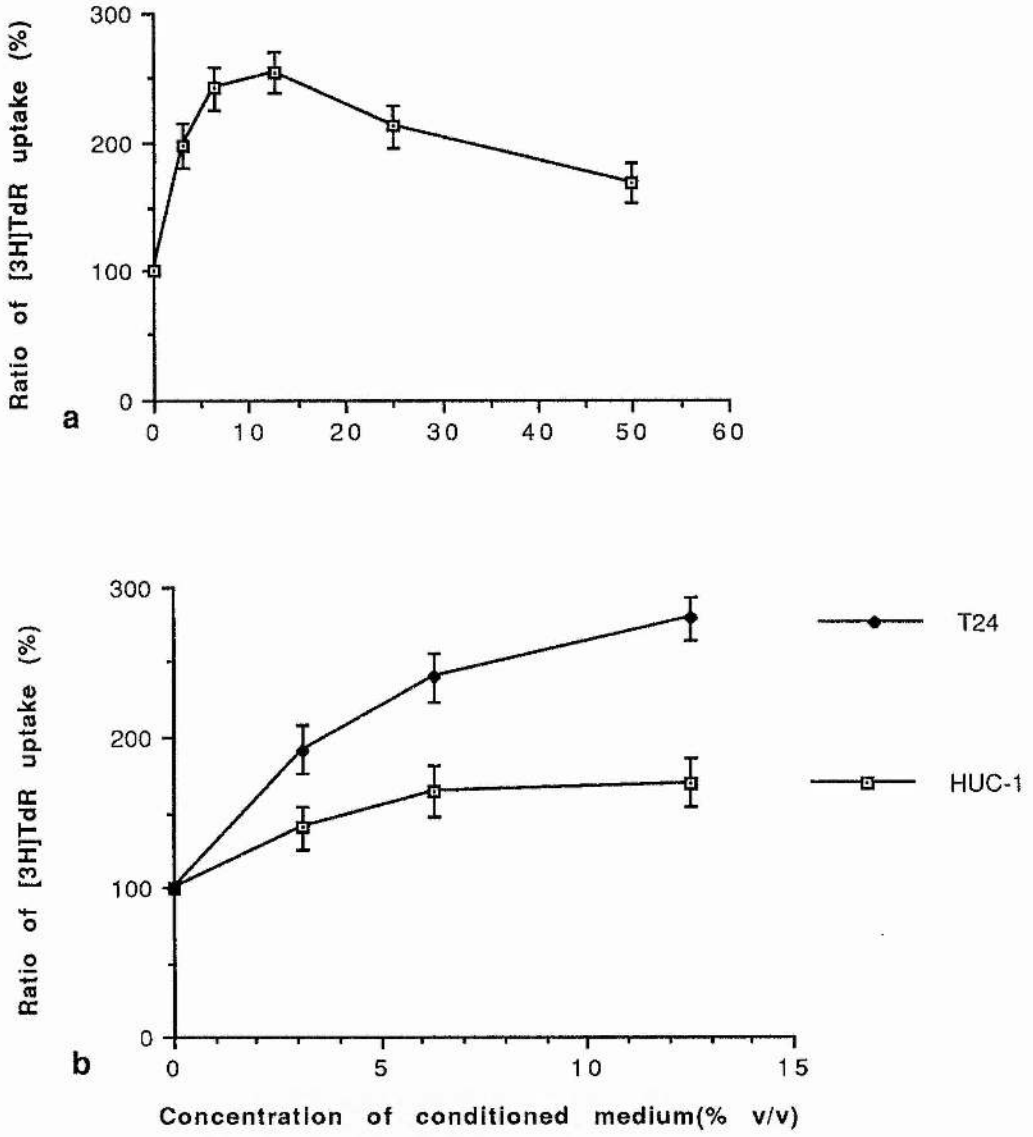


Fig. 3-3-5. Growth-stimulating activity of sff conditioned medium (a) full range (3.1-50 % v/v) of double diluted conditioned medium. (b) stimulating effect toward T24 and HUC-1 cells in the optimal range (3.1-12.5 % v/v). Back ground counts as determined in wells without cells is <4% of control in (a), and <1% in (b). Each point represents a mean of 4 measurements ±SEM.

3-3-3-4 Stem cell factor-like effect in conditioned medium

Murine HPP-CFC responded to growth factor(s) or/and conditioned medium leading to the formation of colonies in soft agar dishes and HPP-CFC derived colonies were defined as those in excess of 2 mm in diameter. Colonies were counted and results are shown in Fig. 3-3-6. rmu SCF or conditioned medium alone had no effect of stimulation of bone marrow cells to form colonies. 4± 1.08 colonies were formed in dishes containing rmu GM-CSF plus rhu M-CSF. While 19±2.12 colonies were formed in dishes containing rmu SCF combined with these two factors. Tumorigenic and non-tumorigenic conditioned medium combined with M and GM-CSF had shown the synergistic effect with an average of 20.25±1.97 and 20.75±1.03 colonies were formed respectively.

A stem cell factor-like synergistic effect was observed with medium conditioned by SV-HUC-1T cells.

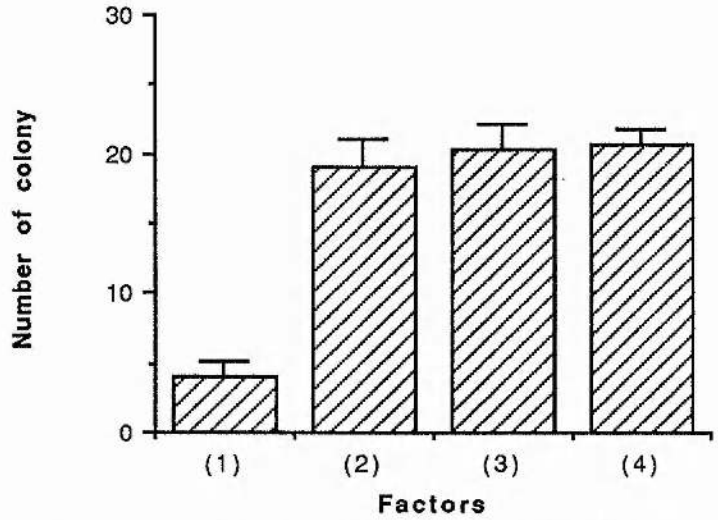


Fig. 3-3-6 Murine bone marrow HPP-CFC assay of stem cell factor-like activity. (1) GM-CSF + M-CSF; [4±1.08]. (2) SCF + GM-CSF + M-CSF; [19±2.12]. (3) Tumorigenic CM + GM-CSF + M-CSF; [20.25±1.97]. (4) Non-tumorigenic CM + GM-CSF + M-CSF; [20.75±1.03]. Bars represent standard errors of mean values obtain from 4 independent samples.

3-3-3-5 Levels of human soluble SCF in conditioned medium

An average 256±43 (±SEM) pg/ml of SCF were detected in medium conditioned by SV-HUC-1T cells (four replicated experiments) and an average of 550±66 (±SEM) pg/ml of SCF were detected in medium condition by SV-HUC-1 cells (three replicated experiments) (Fig. 3-3-7). No cross species reaction of 7% (v/v) fetal calf serum was detected while using both kits.

The quantity of human stem cell factor presented in the conditioned medium of tumorigenic cells is significant less ($p \leq 0.01$) than that of non-tumorigenic cells.

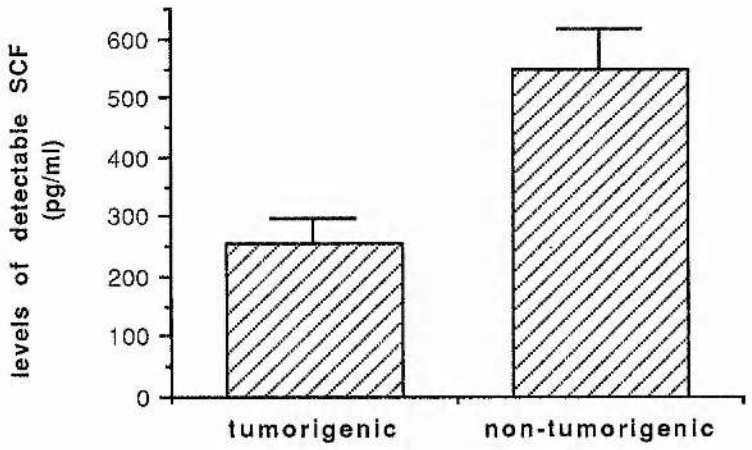


Fig. 3-3-7. Concentration of detectable human stem cell factor in medium conditioned by tumorigenic and non-tumorigenic HUC cells, using ELISA kits.

3-3-3-6 IL1-like activity in conditioned medium

The dose response curve of thymocyte proliferation-stimulating effect of IL1 with a suboptimal dose of ConA (1μg/ml) was shown in Fig. 3-3-8a. The [^{3}H]TdR uptake by murine thymocytes after treating with conditioned medium (four samples for each result) was shown in Fig. 3-3-8b. These results represented 25%(v/v) concentration of conditioned medium and read from equivalent level of uptake in the standard titration curve the estimated final concentration of IL1 α in conditioned medium of tumorigenic and non-tumorigenic cells was 2.8 U/ml and 10.5 U/ml respectively.

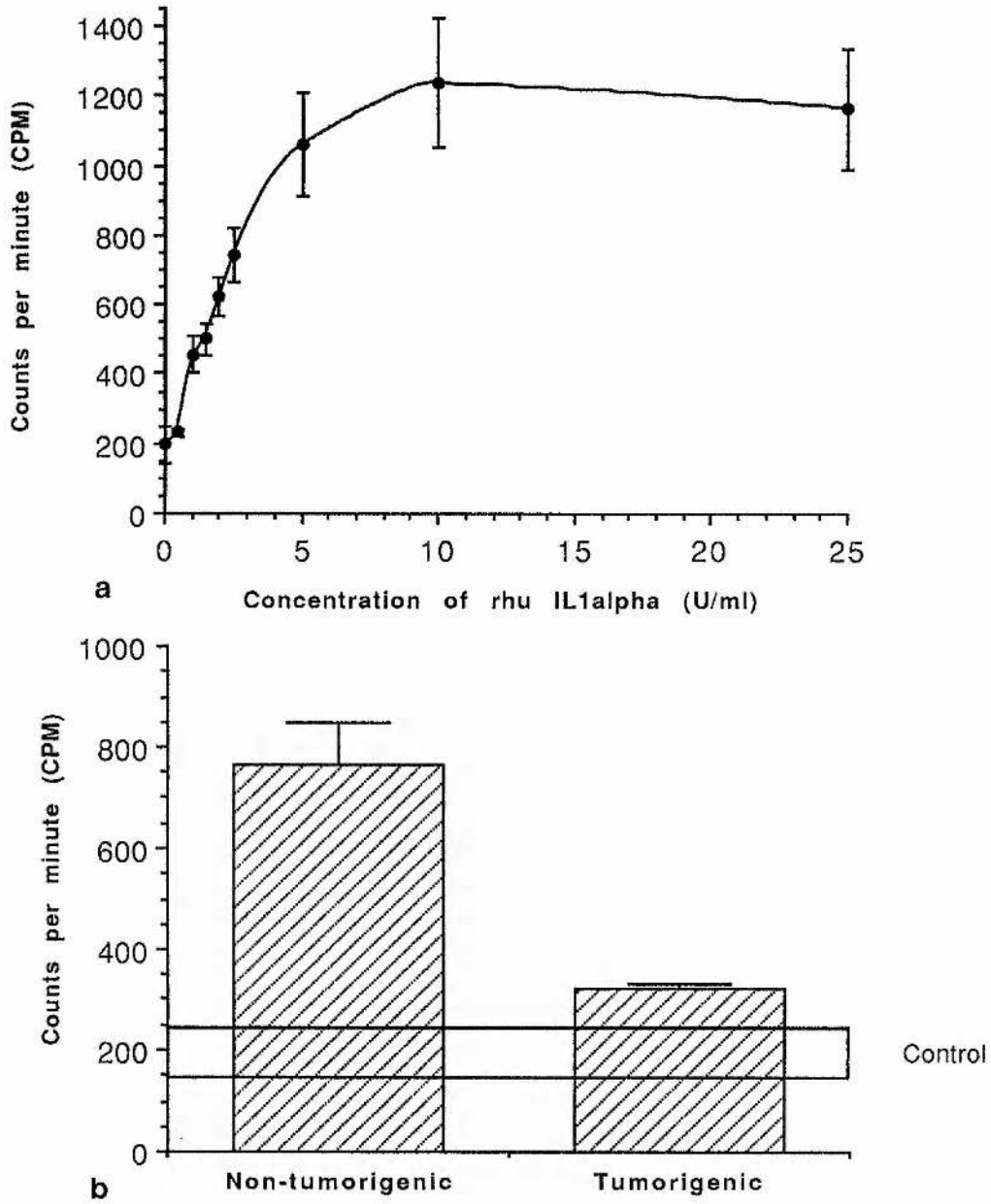


Fig. 3-3-8. Human IL1 α-like activity of conditioned medium. (a) Standard dose response of thymocyte proliferation-stimulating effect of increased concentration of IL1α with a suboptimal dose of ConA(1μg/ml). (b) The level of [3H]TdR uptake of 25%(v/v) concentration of medium conditioned by tumorigenic and non-tumorigenic HUC cells.

3-3-4 Discussion

In the flow cytometry studies the DNA content at G0/G1 peak of tumorigenic cells is different from that of non-tumorigenic cells However their DNA index (1.03) is within the accepted(1±10%) limits of error. (Dressler, L. G. 1990). Tumorigenic cells were not assayed for ploidy as the SV-HUC-1 cells used as a standard are pseudo-diploid.

Post-confluent culture of SV-HUC-1T cells had shown that they had higher saturation densities than SV-HUC-1 cells. The increased density, however is not due to an increase in growth rate during the exponential phase of growth. The doubling time of tumorigenic cells grown in 7% FCS F12 is 40 hours which is similar to the parental non-tumorigenic cell. Thus tumorigenic cells might not have a growth advantage over non-tumorigenic cells while grown in serum supplemented medium.

We have reasons to believe that in the HPP-CFC assay, the synergistic effect of conditioned medium is not solely attributable to SCF. The dose of rmu SCF used in the assay is 20 times higher than the concentration of human SCF in the conditioned medium. However in the HPP-CFC assay the conditioned medium did exhibit a similar synergistic effect. Further more in a different proliferation stimulating assay the result showed that the specific activity of human SCF was 800 fold less than that of rat SCF (Martin, F. H. *et al* 1990).

Data suggesting that SV-HUC-1N cells secrete factors which may support their own proliferation comes from assays where the addition of conditioned medium stimulated the proliferation of both

T24 and SV-HUC-1 cells in sff culture. In addition, this suggests that the action of this factor(s) may be non-cell-specific.

The biological effect of growth factors is mainly dependent on the tyrosine kinase activities of membrane receptors; one might anticipate that constant stimulation of a growth-factor receptor by its ligand would lead to a loss of growth regulation. However this is in contrast to our findings with respect to SCF and IL 1α. The concentration of SCF and the activity of IL 1α were lower in tumorigenic cell conditioned medium. One possibility is that there are less tumorigenic cells to produce the factors. However in Fig. 3-1-3 we have shown that saturation density is higher in tumorigenic cells. Some other explanations for reduced growth factor(s) in tumorigenic cell conditioned medium may be postulated.

A structurally altered receptor that is active in the absence of growth factor, or enhanced cellular responsiveness to a growth factor may result from a change in the number or affinity of the receptors for their growth factor (Sporn, M. B. *et al* 1985). Binding of growth factors is followed rapidly by internalization and degradation of the receptor-ligand complex (Schlessinger, J. *et al* 1978) thus exhausting the growth factor.

Concepts of how receptors function have changed in recent years with the realization that many receptors can give rise to soluble, non-membrane-bound forms and these forms may have a biological role in this regard. Soluble receptors may be formed by proteolytic cleavage of the extacellular domain, or through alternative splicing of mRNA leading to expression of the extracellular region . These soluble forms of receptors have been proposed to act as biological sinks that function to modulate or temper the biological response of their cognate ligand. It may be that either SCF and c-Kit may

function as a ligand, a receptor, or both (Anderson, D. M. *et al* 1990). Levels of this as yet unidentified soluble receptor might increase in tumorigenic cells and trapping the soluble growth factor leading to a lower detectable concentration. Similarly, several growth factors are produced as transmembrane proteins that can be released by specific proteolytic cleavage to generate free factors (Pandiella, A. *et al* 1992). In general, the production of growth factors as cell surface molecules may permit a set of direct cell-cell interactions that have a degree of spatial organization that could not easily be achieved with freely diffusible factors. These interactions may be important in processes of normal development, including cellular migration and homing, and could also be involved in related disease processes such as tumor metastasis (Massague, J. 1990). Another function of this form of growth factor may be to prevent the molecule from diffusing away from the sites of synthesis and thereby ensuring high enough local concentrations for activation of the responsive cells (Flanagan, T. G. 1991). Alteration in the balance between the diffusible and membrane-bound forms of growth factors may lead to phenotypic abnormalities (Majumdar, M. K. *et al* 1994) as seen in Alzheimer's disease (Sisodia, S. S. *et al* 1990). IL 1α and SCF are biologically active in both membrane-bound and soluble form (Anderson, D. M. *et al* 1990; Conlon, P. J. *et al* 1987). In this case the cell may have an increase in the level of the membrane-bound form with a decrease in the level of the soluble form of growth factors leading to tumorigenic transformation.

Conclusion

We have established a system in which tumorigenic cell and non-tumorigenic cells share a similar DNA fingerprinting pattern. Using this system, any molecular or biological difference which appear between the two cells type may represent changes attributable to tumorigenicity and not merely individual variation.

Furthermore a human urothelial cell line which can be continuously cultured in sff condition is established. Using this line to assay the growth regulation effect of a factor will eradicate the uncertainty caused by the serum components in culture medium.

In our experiment, we found that cells could be transformed in a serum free and growth factor free situation. This model may give a possible explanation to a clinical observation of the heterogeneity (metastasis, drug sensitivity) of a cancer: cells were transformed in an area (area around central necrosis) where serum components were deprivated and then expressed in a different phenotype result in the heterogeneity.

The level of soluble human stem factor and inter-leukin 1a are found to be lower in medium conditioned by the tumorigenic cell than by the non-tumorigenic cell. The proposed explanation for this observation involves both soluble and membrane bound forms of growth factors and/or receptors. Future work could concentrate on assaying pre and post-translation levels of both forms of growth factor. Similarly possible identification if the putative soluble form receptors may help to clarify the mechanism involved in this finding.

Chapter IV

Roussin's black salt a novel nitric oxide donor acts as a cytotoxic agent

Summary

Outline

4-1 Cytotoxic effect of Roussin's black salt

4-1-1 Introduction

4-1-2 Procedures

4-1-2-1 Growth inhibitory effect of RBS on expoenetially growing cells

4-1-2-2 Growth inhibitory effect of laser-irradiated RBS on exponential growing cells

4-1-2-3 Heamoglobin suppression of the growth inhibitory effect of RBS

4-1-2-4 Turnbull reaction of RBS-treated cells

4-1-3 Result

4-1-3-1 Cytotoxic effect of RBS

4-1-3-2 Photosensitivity of RBS

4-1-3-3 Heamoglobin suppressed the cytotoxic effect of RBS

4-1-3-4 Turnbull reaction of RBS-treated cells

4-1-4 Discussion

4-2 Roussin's black salt causes DNA damage within target cells

4-2-1 Introduction

4-2-2 Procedures

4-2-2-1 Colony formation assay of CHO/xrs cells to quantatively assess cytotoxicity of RBS

4-2-2-2 Micronuclei assay of RBS treated CHO/xrs cells

4-2-3 Result

4-2-3-1 Cytotoxicity of RBS on CHO/xrs cells

4-2-3-2 Micronuclei assay of RBS treated CHO/xrs cells

4-2-4 Discussion

Conclusion

Fig 4-1-1 Growth ratio of SV-HUC-1 cells after treatment with RBS for 1 hour. At a range of 1μM to 10μM, cells have 1 to 99% of growth inhibition.

Fig 4-1-2 Growth ratio of SV-HUC-1 and T24 cells after treatment with different concentrations of RBS for 1 hour. ID_{50}=5.16μM for SV-HUC-1 and ID_{50}=4.96μM for T24 cells.

Fig 4-1-3 Growth ratio of SV-HUC-1 cells after treatment with 4μM and 7μM RBS for lengths of time varying from 30 to 240 minutes.

Fig 4-1-4 Growth ratio of SV-HUC-1 and T24 cells after treatment with different concentrations of Thiotepa for 1 hour.

Fig 4-1-5 Growth ratio of 1μM RBS-treated T24 cells after either irradiation or deprivation from light for 1 hour.

Fig 4-1-6 Growth ratio of T24 cells after treatment with different concentrations of RBS and either irradiated with 5mW of laser light (λ=457.9 nm) or light deprivation for 1 hour.

Fig 4-1-7 Growth ratio of 2μM or 3μM RBS-treated T24 cells after irradiation with different intensities of laser light (λ=457.9 nm) for 40 minutes.

Fig 4-1-8 Growth ratio of RBS-treated T24 cells with or without haemoglobin. (a) Cells were treated with 7μM RBS and kept in dark for 1 hour. (b) The same treatment as in (a)but with 1 hour light exposure. (c) Cells were treated with 50μM AB-RBS and exposed to light for 1 hour.

Fig 4-1-9 Photograph of column chromatography after run through of solution containing (A) Bovine albumin and RBS. (B) RBS. (C) Bovine haemoglobin and RBS. Arrow head indicate that RBS was held up by resin. RBS bound to albumin and haemoglobins thus run through the column.

Fig 4-1-10 Growth ratio of 50μM AB-RBS-treated T24 cells with or without 5μM haemoglobin after irradiation with different intensities of laser light (λ=457.9 nm) for 40 minutes.

Fig 4-1-11 RBS-treated T24 cells after the Turnbull reaction. Blue color indicated bound ferrous salt. Bar=1.1μm.

Fig 4-2-1 Survival of CHO and xrs cells after treated with different concentrations of RBS for 1 hour.

Fig 4-2-2 Number of micronuclei per 100 binuclear cells. CHO or xrs cells were pretreated with 10 μM RBS for 40 minutes, cytokinesis was blocked with 3μg/ml of Cyto B. Controls were the same but without RBS pretreatment. Two separate experiments were performed and 3 samples were determined in each experiment.

summary

We report the cytotoxicity of nitric oxide (NO) against human urothelial cells *in vitro* and propose a possible mechanism for this activity. In the human body NO has been identified as a blood vessel dilator, an inter-neuronal messenger and a cytotoxic agent against bacteria and tumor cells.

An iron-sulphur cluster nitrosyl "heptonitrosyl-tri-μ3-thioxotetraferrate" (Roussin's black salt, RBS) is a generator of NO. The cytotoxicity of RBS has been investigated using two human urothelial cell lines: SV-HUC-1 (derived from normal tissue) and T24 (derived from cancerous tissue). Both cell lines demonstrate a dose- and contact time- dependency for RBS cytotoxicity. Growth inhibition of cell induced by RBS was increased in the presence of light, indicating a photosensitivity of RBS, and reduced in the presence of hemoglobin.

Chinese hamster ovary (CH0) cells and a CHO mutant, deficient in DNA repair (xrs-5), were used to investigate the possible mechanism of action of NO. The xrs-5 cell line proved to be significantly more sensitive to the cytotoxicity of RBS than the CHO cell line, a difference which correlated with a significantly higher number of micronuclei present in RBS-exposed xrs-5 cells. These data suggest that RBS cytotoxicity may involve DNA damage.

Although *in vivo* studies need to be performed to assess the potential of RBS as a chemotherapeutic agent, current findings suggest that chemicals which release NO may have a role in cancer treatment.

Outline

In this chapter, the potential cytotoxic effect of a novel nitric oxide donor-"RBS" is discussed in 2 sections.

In the first section, **cytotoxic effect of RBS**, the growth inhibitory effect of RBS related to the drug concentration and exposure time is examined. The effect of light irradiation and haemoglobin on the cytotoxic effect of RBS is illustrated.

In the 2nd section, **RBS causes DNA damage within target cells**, the evidence to show that RBS causes DNA damage within cells is probed by using CHO and its DNA repair deficient mutant xrs-5 cells.

4-1 Cytotoxic effect of RBS

4-1-1 Introduction

There are two main ways to eradicate cancer from a patient: locally and systemically. Traditionally cancer was locally treated with surgery and radiotherapy before the option of systemic treatment with chemical compounds became available. A brief clinical remission in a patient with lymphoma given nitrogen mustard during World War II was the first documented clinical use of chemotherapy.

Anticancer drugs may be classified into a number of families based on either the mode of action or their origin. These include alkylating agents, antimetabolites and natural products that have anticancer activity. Alkylating agents are chemically diverse drugs which may produce an intermediate form containing electron deficient, reactive alkyl groups. These drugs undergo a process known as alkylation in which the reactive alkyl group covalently binds with chemical groups on biological molecules that have an excess of electrons. This results in a cytotoxic effect on target cells. Alkylation of bases in DNA appears to be the major cause of lethal toxicity.

Antimetabolites, are compounds which mimic the structure of normal metabolites. They may compete as substrates for enzyme activity leading to the inhibition of critical biochemical pathways. Most drugs in this family inhibit nucleic acid synthesis either directly or indirectly and tend to be cell cycle dependent.

Other chemotherapeutic agents are drawn from a wide variety of sources like anitbiotics, hormones or plant extract. Some of these

were discovered by pure chance and their origin gives little indication as to their potential activity against a particular tumor.

In chemotherapy an important concept is that a given dose or course of therapy will kill a constant fraction of the cell population rather than a constant number of cells and gives a greater fractional cell-kill to more rapid growing tumor (Gregory, W. M. 1992). Chemotherapeutic agents damage not only tumor cells but normal tissue, the only reason that chemotherapy is feasible is that normal tissue may recover sometimes more rapidly than the tumor cells.

Nitric oxide (NO) has been a hotspot of research in recent years and is a natural product accounting for several separate biological functions within the human body. NO had been identified as a vessel dilator. As representing a completely novel class of neuronal messenger. Also as an effector molecule released by murine macrophages and other cells after immunological activation. At present the only clearly established role for NO is as a cytotoxic molecule against invading microorganisms and tumor cells. Several mechanisms for the cytotoxic effect of NO have been proposed including: toxic free radical, inhibition of mitochondrial respiration, iron loss, inhibition of DNA synthesis and DNA damage.

Roussin's black salt (RBS) has been demonstrated to be a novel NO donor. It contains seven nitrosyl groups linked to an iron-sulphur framework, once in solution it will decomposed to release NO. The vasodilatory effect of RBS is blocked by hemoglobin and accelerated during exposure to light (λ = 514.5 and 457.9 nm).The cytotoxicity of RBS has been investigated using two human urothelial cell lines: SV-HUC-1 (derived from normal tissue) and T24 (derived from cancerous tissue). Thiotepa, an alkylating agent, is widely used for local treatment of bladder cancer. For the purpose of comparison,

the cytotoxicity of thiotepa also was performed using both phenotypes of human urothelial cells.

4-1-2 Procedures

4-1-2-1 Growth inhibitory effects of RBS on exponentially growing cells

$1x10^5$ cells/flask of T24 cells or $1.5x10^5$ cells/flask of SV-HUC-1 cells were seeded in 25 cm^2 flasks with 5 ml of serum supplemented medium. Cells were incubated under standard conditions for 48 hours and allowed to attach and achieve exponential growth. The supernatant was replaced with 4.5 ml of serum free medium after two rinses with PBS The whole flask (except cap) was covered with tin foil to exclude light. 0.5 ml of ten times final concentration of drug (RBS) dissolved in PBS was added (0.5 ml of PBS only for control group) to begin the assay. All assay procedures were performed in a darkened room and a lamp (40W) filtered with ILFORD 904 safe light glass was used as an illumination source. After a 60-minute (or designated period) incubation at 37°C and the medium containing the drug was sucked out and the tin foil cover was removed. Flasks were rinsed twice with PBS and 5 mls of fresh serum supplemented culture medium added. Incubation was continued for a period of at least two cell doubling times (2 days for T24 cells, 4 days for SV-HUC-1 cells). During this period the cells in the control group (PBS-treated) were in exponential growth. Cells in all treatment groups were dispersed with trypsin and diluted in serum supplemented medium. The total volume of cell suspension present was important to allow the calculation of the total number of viable cells. Viable

cells ,which did not stain green, were counted with a hemocytometer after staining cells for 10 minutes with 2% fast green [2-16]. Results were expressed as the ratio of cell growth, defined by the ratio of viable cells in drug treated populations to non-drug treated populations.

0.15M NaCl solution was used to dissolve drug and as culture fluid during the cytotoxicity assay of "Thiotepa".

98% of SV-HUC-1 cells which were incubated for 30 minutes in a 65°C water bath were positively stained with fast green thus this was used as a non-viable cell control. For each growth inhibition curve, flasks were counted in triplicate for each treatment.

4-1-2-2 Growth inhibitory effects of laser-irradiated RBS on exponentially growing cells

Laser irradiations were conducted in a laser equipped laboratory which was darkened. A red safelight (60W) was used as the sole means of illumination. An Argon ion laser (Spectra Physics Ltd., type 168-09) was the source of irradiated light and 457.9nm wave length light used.

The procedures were the same as above but PBS instead of serum free medium was used as culture fluid for the period of drug exposure. This was to avoid phenol red in the medium interfering with light absorption by RBS. A selected intensity of laser light was used to irradiate the cells and tin foil was removed during that period. The control group received only PBS.

4-1-2-3 Hemoglobin suppression of the growth inhibitory effects of RBS

Ferro-haemoglobin was prepared as mentioned in [2-18-1] The procedures were the same as above with the addition of ferro-haemoglobin solution just before cells were irradiated with laser

light. Controls were treated identically but no drugs were added. The absorbance of light by the solution was determined by a double beam spectrophotometer.(Cecil, CE594, UK).

4-1-2-4 Turnbull reaction of RBS-treated cells

$5x10^4$ T24 cells were grown on a glass slide overnight. Cells were treated with 10^{-3} M RBS (without RBS treatment as control). They then underwent the Turnbull reaction [2-21].

4-1-3 Result

4-1-3-1 Growth inhibitory effects of drugs on exponentially growing cells

Experiments were performed to determine if the cytotoxic effects caused by exposing growing cultures of human urothelial cells to RBS could be quantitatively and reproducibly assessed using different cell phenotypes (tumorigenic: T24, non-tumorigenic: SV-HUC-1).

In experiments where a concentration of 10μM RBS or more was used very few viable cells could be counted. However, if the concentration used was less than 1μM then no obvious cytotoxic effects on cells were shown (Fig. 4-1-1). Thus a range of concentrations between 1μM to 10μM of RBS was used in these assays.

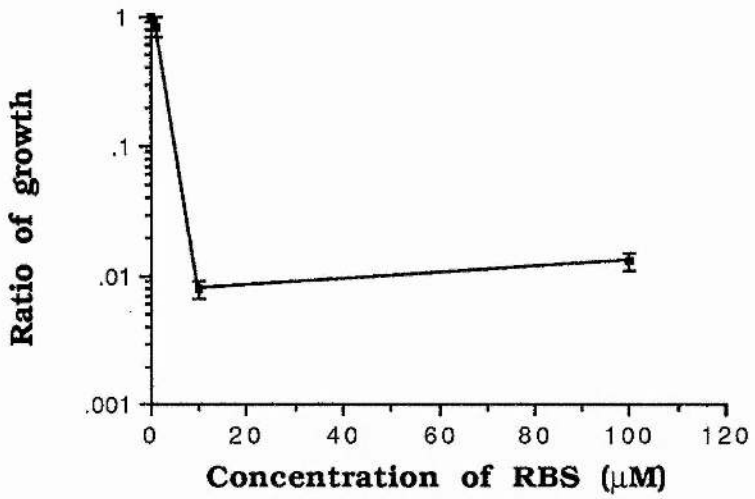


Fig. 4-1-1 Growth ratio of SV-HUC-1 cells after treatment with RBS for 1 hour. At a range of 1μM to 10μM, cells have 1 to 99% of growth inhibition.

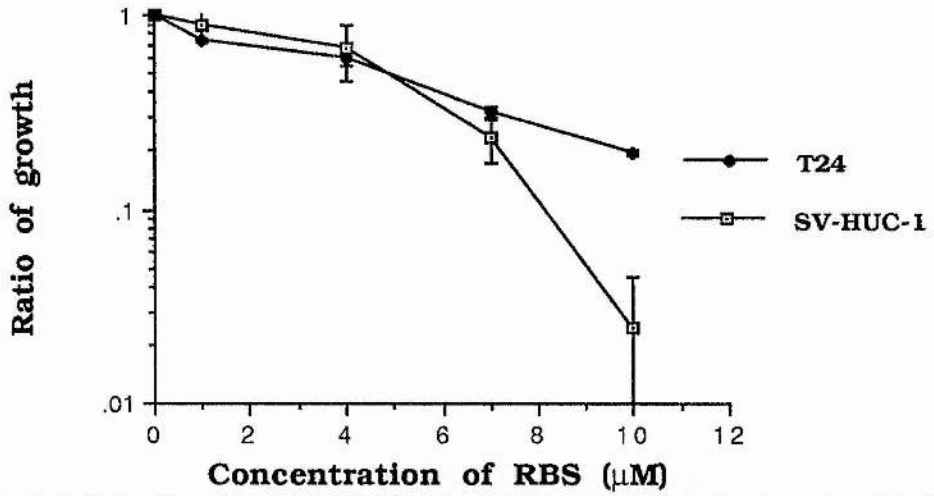


Fig. 4-1-2 Growth ratio of SV-HUC-1 and T24 cells after treatment with different concentrations of RBS for 1 hour. ID_{50}=5.16μM for SV-HUC-1 and ID_{50}=4.96μM for T24 cells.

Cells were exposed to different concentrations of RBS for 60 minutes and results showed that growth inhibition in both human urothelial cell phenotypes was dependent on concentration. The responses were similar with ID_{50}=5.16μM for SV-HUC-1 and

ID_{50}=4.96μM for T24 cells (Fig. 4-1-2). The ID50 was defined as the concentration of drug which inhibited growth of the target cells by 50%.

The length of time cells were exposed to the drug was a determinant factor of the growth inhibitory effect of RBS. Increasing the exposure period of SV-HUC-1 cells to 4μM of RBS from 60 minutes to 120 minutes resulted in a decreased growth ratio relative to controls of 0.5 to 0.25. Similarly for SV-HUC-1 cells increased exposure time for higher concentrations of RBS resulted in greater growth inhibition. For example, the relative growth ratio of SV-HUC-1 cells after a 60 minute exposure to 7μM of RBS was 0.2, whilst the same cells exposed to 7μM of RBS for 120 minutes showed only a 0.01 relative growth ratio (Fig. 4-1-3).

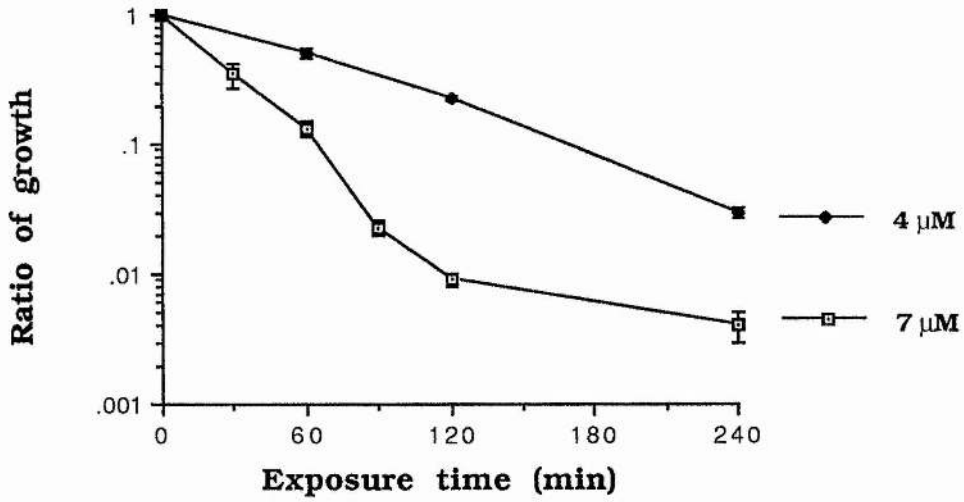


Fig. 4-1-3 Growth ratio of SV-HUC-1 cells after treatment with 4μM and 7μM RBS for lengths of time varying from 30 to 240 minutes.

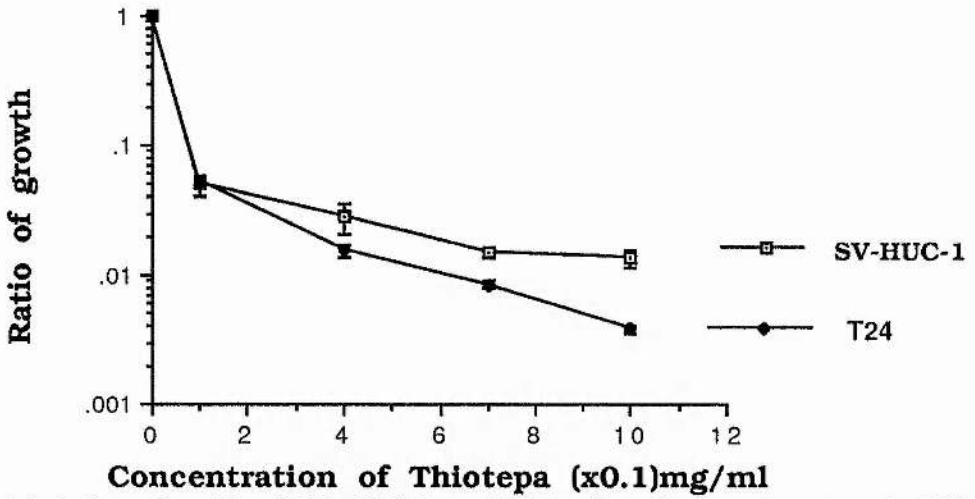


Fig. 4-1-4 Growth ratio of SV-HUC-1 and T24 cells after treatment with different concentrations of Thiotepa for 1 hour.

Thiotepa is used as an anticancer drug with a concentration of 1mg/ml in the clinical treatment of bladder cancer. A range of concentrations of 0.1mg/ml to 1mg/ml of thiotepa was used to assess the cytotoxic effect of this drug under the same experimental condition used to test the toxicity of RBS. Results showed that the inhibition of growth of human urothelial cells after exposure to thiotepa was dose-dependent. Similar responses were observed in both tumorigenic and non-tumorigenic cells (Fig. 4-1-4).

4-1-3-2 Photosensitivity of RBS

T24 cells were used as target cells to examine the photosensitivity of RBS. Cells were treated with different concentrations of the drug and either exposed to light at a distance of 150 cm from an 80W fluorescent light, or kept in dark (as a comparison) for 1 hour. Viable cells were counted at the defined end point. By comparing

the growth ratio of cells irradiated with light relative to cells kept in the dark it could be demonstrated that light enhanced the cytotoxic effect of RBS. Results in Fig. 4-1-5 show a significantly enhanced cytotoxic effect was observed in cells treated with 1μM of RBS and irradiated with light ($p<0.01$). No viable cells were found under the same conditions when cells were treated with 10μM of RBS (data not shown).

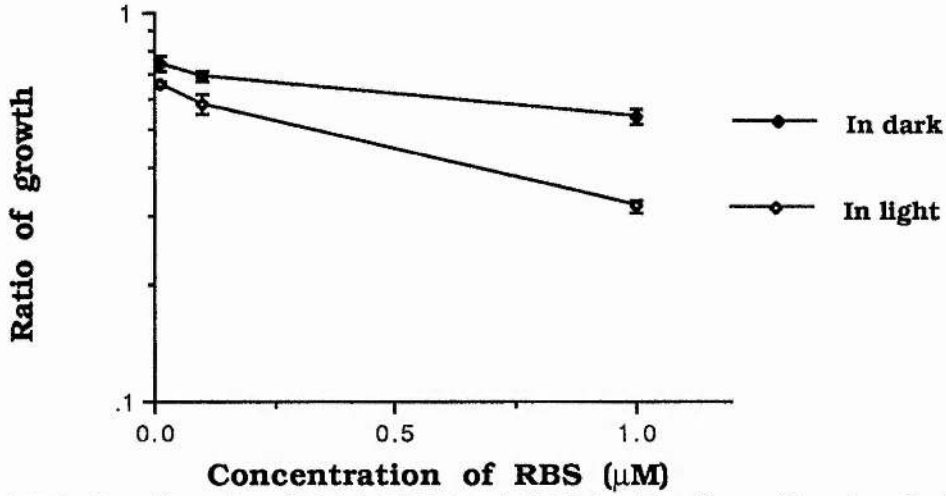


Fig. 4-1-5 Growth ratio of 1μM RBS-treated T24 cells after either irradiation or deprivation from light for 1 hour.

To control the parameters of light intensity and wave length, assays were performed in a laser light equiped and darkened laboratory. Light with a wave length of 457.9 nm was chosen for its optimal absorption by RBS with the particular laser available (Appendix III personal communication with Dr. I. Megson). It was directed via a silver front mirror creating a 45 cm^2 irradiation field where culture specimens were located. This light itself had no obvious growth inhibitory effect on cells at intensities of up to 120 mW (data not shown).

Cells were treated with different concentrations of RBS and irradiated with an intensity of 5mW of specified light for 40 minutes or kept in dark for the same period. Viable cells were counted at the defined endpoint [4-1-2] and the ratio of growth relative to controls plotted. Results showed that the cytotoxic effect of RBS was significantly enhanced by irradiating with light (Fig. 4-1-6). For example the growth ratio of cells treated with 4 μM RBS and kept in dark was 0.1. A decrease to 0.02 was found for cells treated with an identical concentration of RBS but irradiated with 5mW light.

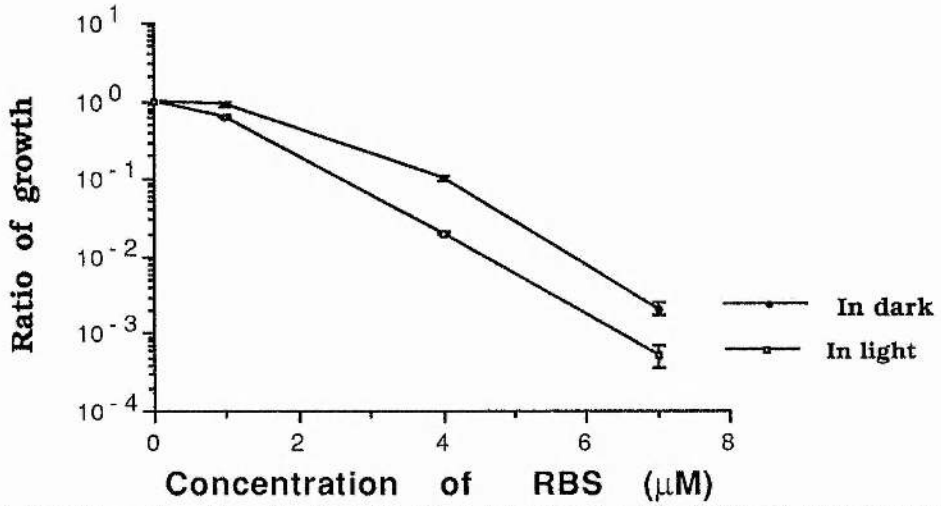


Fig. 4-1-6 Growth ratio of T24 cells after treatment with different concentrations of RBS and either irradiated with 5mW of laser light (λ=457.9 nm) or light deprivation for 1 hour.

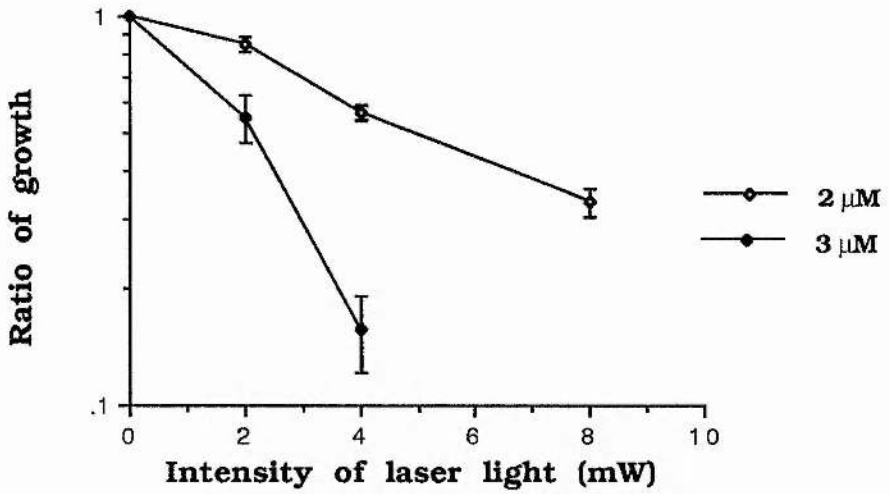


Fig. 4-1-7 Growth ratio of 2μM or 3μM RBS-treated T24 cells after irradiation with different intensities of laser light (λ=457.9 nm) for 40 minutes.

The intensity of light which the cells were irradiated, influenced the inhibitory effect of RBS. For T24 cells given 2μM of RBS increasing the intensity of light from 2mW to 8 mW result in a decrease in growth ratio relative to controls of 0.84 to 0.33 (Fig. 4-1-7). The same observation i.e an increase in growth inhibition was made with respect to increased intensities of light for cells treated with different concentrations of RBS (Fig. 4-1-7).

4-1-3-3 Haemoglobin suppression of the growth inhibitory effects of RBS

Haemoglobin is a scavenger of NO and has been shown to block the vaso-dilatory effects of RBS. Several experiments were performed to establish whether haemoglobin might prevent the cytotoxic effect of RBS.

Because vertebrate haemoglobins consist of 4 heme containing polypeptide chains and RBS contains seven nitrosyl groups linked

to an iron-sulphur framework, double the concentration of haemoglobin to RBS was used in these experiments.

T24 cells were treated with 7μM of RBS for 60 minutes. The cytotoxic effect of RBS was markedly reduced by the addition of 15 μM of bovine haemoglobin (Fig. 4-1-8a). The same observation was made when specimens were exposed to light (Fig. 4-1-8b).

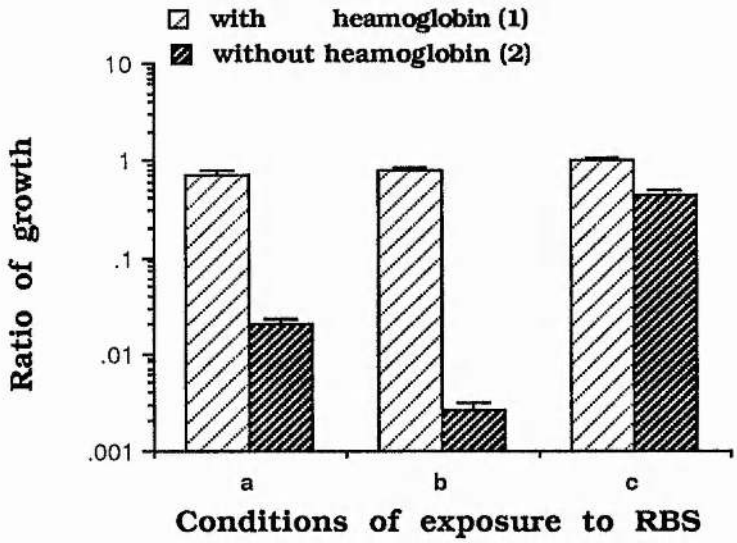


Fig. 4-1-8 Growth ratio of RBS-treated T24 cells with or without haemoglobin. (a) Cells were treated with 7μM RBS and kept in dark for 1 hour. (b) The same treatment as in (a)but with 1 hour light exposure. (c) Cells were treated with 50μM AB-RBS and exposed to light for 1 hour.

However, by using a column gel-chromotograph (column PD-10, Pharmacia, Sweden) analysis showed that molecules of RBS were tightly bound to both haemoglobin and bovine albumin (Fig. 4-1-9).

Cytotoxicity of albumin bound RBS (AB-RBS) was thus assessed.

The growth ratio of cells treated with 50μM of AB-RBS and exposed to light was 2 log higher than cells which were treated with 7μM of unbound RBS (Fig. 4-1-8 b2/c2). Thus albumin itself may protect cells against the cytotoxic effect of RBS.

Similarly, haemoglobin had a additional protective effect on cells treated with AB-RBS and irradiated with light (Fig. 4-1-8c)

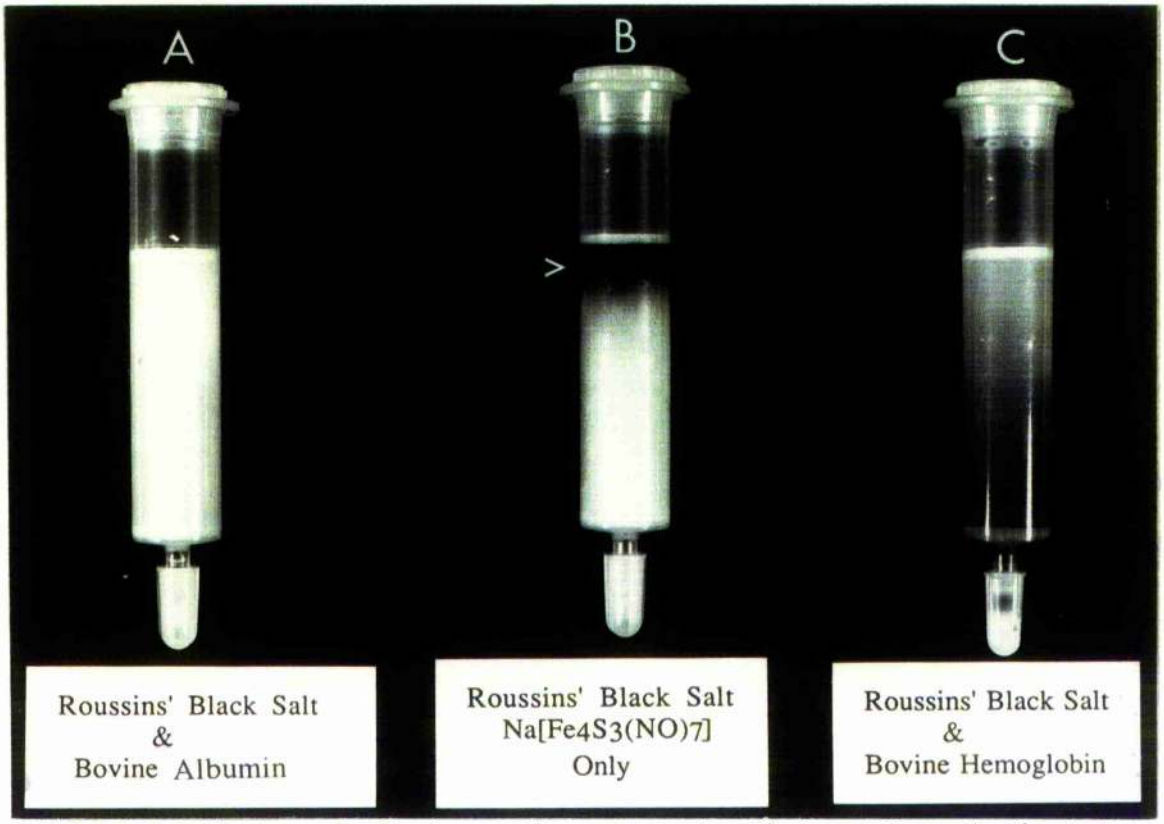


Fig. 4-1-9 Photograph of column chromatography after run through of solution containing (A) Bovine albumin and RBS. (B) RBS. (C) Bovine haemoglobin and RBS. Arrow head indicate that RBS was held up by resin. RBS bound to albumin and haemoglobins thus run through the column.

In further tests haemoglobin suppressed the cytotoxicity of AB-RBS. Cells were treated with 50μM of AB-RBS then exposed to different intensities of 457.9 nm wave length laser light. A concentration of 5μM of haemoglobin was used in this experiment because it permitted a significant amount of light to transmit through solution. By using a spectrophotometer it was determined that 41.6% of the light was absorbed by an 1 cm depth of 5μM haemoglobin. Thus in a

depth of 2 mm (solution depth in the experiment) the absorbance will be 8.3% with 91.7% of light passing through solution and reaching the cells. To ensure that the same intensity of light was delivered to cells in the solution containing 5 μM haemoglobin and cells in the solution without haemoglobin different intensities of light were used. The cells in haemoglobin received a 120mW of light whilst these cells in solution without haemoglobin received 110mW of light (120x91.7%=110). By comparing the relative growth ratio for these cells it was found that haemoglobin significantly ($p \leq 0.05$) decreased the cytotoxicity of AB-RBS (Fig. 4-1-10).

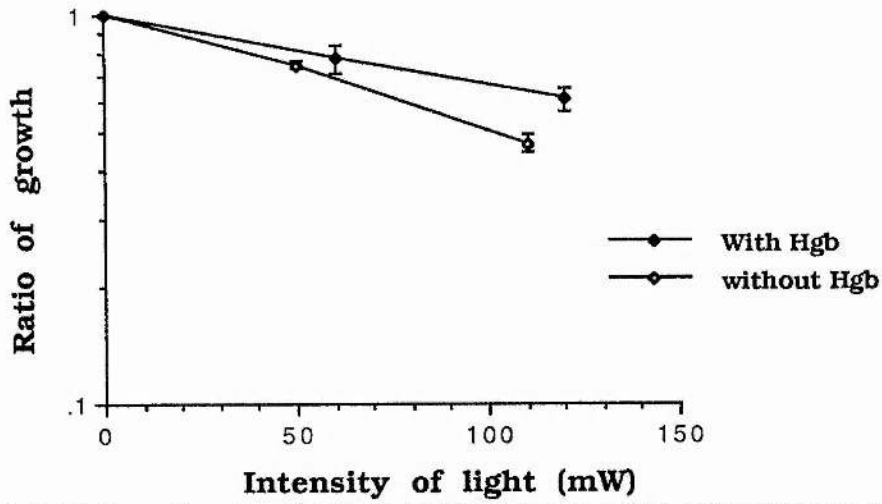


Fig. 4-1-10 Growth ratio of 50μM AB-RBS-treated T24 cells with or without 5μM haemoglobin after irradiation with different intensities of laser light (λ=457.9 nm) for 40 minutes.

4-1-3-4 Turnbull reaction of RBS-treated cells

In Fig. 4-1-11, it can be seen that RBS-treated cells became blue (while control cells didn't change color) after the Turnbull reaction. This indicated that ferrous salt had bound directly to the RBS-

treated cells. Because RBS itself is a ferrous salt then this binding ferrous salt might be the RBS.

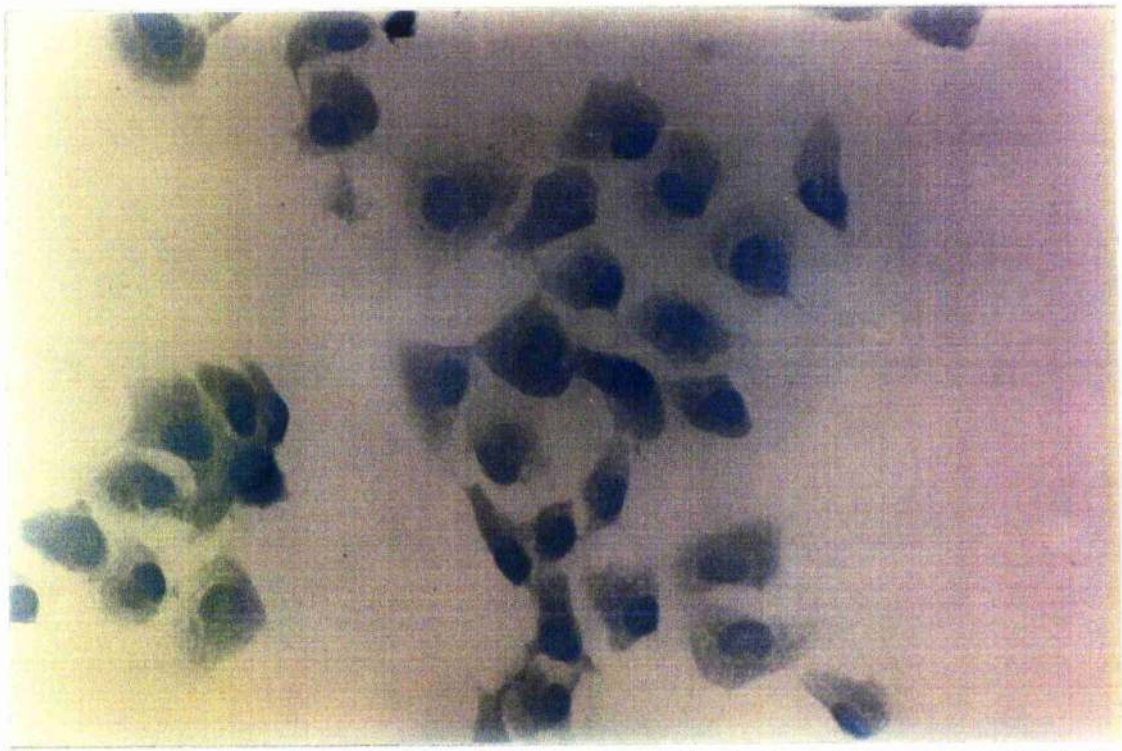


Fig. 4-1-11 RBS-treated T24 cells after the Turnbull reaction. Blue color indicated bound ferrous salt. Bar =1.1 μm.

4-1-4 Discussion

New anticancer agents need to be tested *in vitro* to demonstrate their cellular toxicity before further *in vivo* investigations are performed. There are several methods to assess the cellular damages caused by anticancer drugs and these can be divided into clonogenic and nonclonogenic assays.

Clonogenic assays

These are used to measure cell survival after drug treatment. Cells are exposed to the drug, followed by plating at different dilutions in Petri dishes and incubation for a period of time (usually 2 weeks). Colonies which form on the dishes represent the surviving fraction of the colony forming cells (CFC). Drug effects can be estimated from the survival rate of CFC. Colony forming cells have a very large potential for proliferation and their lethality represents the cure or long term remission of the tumor with treatment (Roper, P. R. *et al* 1976). Requirements of this assay are the production of a single cell suspension and that the cells demonstrate a high plating efficiency (Bhuyan, B. K. *et al* 1976). In addition, a proportional relationship between the number of cells plated and the number of colonies growing in the non drug-treated group should exist (Hamburger, A. W. 1981).

Nonclonogenic assays

DNA precursor uptake assays that measure radioactive DNA precursor incorporation can be used as an index correlating inhibition of DNA synthesis with cell death. Drug effects are estimated from the changed rate of incorporation of radioactive precursors into DNA. It can however be under estimated due to the drug-induced depression of labeled nucleoside transport or

changes in nucleotide pools, and over estimated due to the blocking of *de novo* pathway in the presence of antimetabolites of DNA precursors [eg. 5-fluorouracil (5FU)] and enhanced utilization of exogenous labeled nucleoside (Miyazaki, N. *et al* 1992). The use of these techniques with compounds whose mechanism(s) of action are not known may not be feasible (Hamburger, A. W. 1981).

The tetrazolium assay, which measures the inhibition of cellular metabolism, is based on the ability of live cells to reduce a tetrazolium-based compound [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) to a blue formazan product (Black, M. M. *et al* 1953). Drug effects were calculated from the percent change of light absorbance. This assay does not differentiate between cytostatic and cytocidal effects on cells and a high background value will exist if medium containing serum or phenol red is used. The assay is limited by the fact that maximal absorbance for many human tumor cell lines is approximately 1.0 absorbance unit (Carmichael, J. *et al* 1987). Chemical reduction of the tetrazolium by the test agents, chemical interference with cellular reduction of tetrazolium (Alley, M. C. *et al* 1988) and a variety of metabolic conditions (eg. depletion of glucose, pH changes) (Vistica, D. T. *et al* 1989) which may alter the production of formazan from tetrazolium can lead to an inaccurate determination of the quantity of living cells (Rubinstein, L. V. *et al* 1990).

A screening assay, used by the National Institute of Health (NIH) in the United States, is used as the initial assessment of drugs against a panel of human tumor cell lines, using automated staining with sulforhodomine-B (SRB) to assess the number of viable cells after drug induced cytotoxicity. SRB is a bright pink aminoxanthene dye

with 2 sulfonic groups and is used as a protein stain. It binds to basic amino acid residues of protein in TCA-fixed cells to provide a sensitive index of cellular protein content hence a sensitive method for measuring drug cytotoxicity in culture. Background levels of RBS staining are sensitive to the length of TCA fixation and serum concentration in the culture medium (Skehan, P. *et al* 1990), in addition it stains dead non-lysed cells as well , so it may over estimate the surviving fraction of cells (Rubinstein, L. V. *et al* 1990). In vital stain assays, dye exclusion is used as an indicator of cell membrane integrity. As the membranes of cells become permeable to dyes they almost certainly lose their clonogenic capacity, however a large number of lethally damaged cells may appear "normal" and may exclude dyes for a short interval after treatment. L. M. Weisenthal (1983a) used fast green as a vital stain to estimate drug cytotoxicity from the change in the ratio of living cells.to an internal standard. He assessed the drug-induced cytotoxicity several days after drug exposure thereby allowing for the expression of lethal damage and the proliferation of surviving cells. Data obtained by this method correlates well with that from clonogenic assays (Weisenthal, L. M. *et al* 1983b, Reznikoff, C. A. *et al* 1986).

If cell lines are to be used for preclinical drug screening it is necessary that *in vitro* drug sensitivities remain relatively stable in long-term culture. The T24 cell line is reported to have a stable drug response in long-term culture, therefore providing a representative model of human bladder cancer and an economical system for estimating the cytotoxic activity of drugs in preclinical studies (Masters, J. R. W. *et al* 1986). Another cell line investigated in this project, SV-HUC-1, have a plating efficiency of approximately 9% at 1000 cells per dish, however, no consistent

proportional relationship could be found between the number of cells plated and the number of colonies observed. This observation may be due to difficulties associated with the production of a single cell suspension (Finlay, G. J. 1984). It is very difficult to accurately manipulate the 96 well plate in the dark so it was decided to use the fast green vital stain assay to determine the inhibition of cell proliferation induced by RBS. The total number of living cells is counted therefore there is no internal standard (eg. duck red blood cell) used and because there is no need to distinguish between tumor and nontumor cells no counter-staining is required.

By using viable cells counted 2 cell doubling times after cells were treated with drugs as the end point, the cytotoxicity of RBS was assessed.

The cytotoxic responses of human urothelium to RBS were related to the drug concentrations (Fig. 4-1-2) and to the duration of exposure (Fig. 4-1-3).

The growth inhibition of urothelium from different donors after exposure to thiotepa (Fig. 4-1-4) or RBS (Fig. 4-1-2) were similar. This result was not unexpected as thiotepa is a direct-acting alkylating agent and therefore metabolic differences in bioactivation between cells is not a factor (Reznikoff, C. A. *et al* 1986). Further more, cells were treated with RBS for 1 hour in most cytotoxicity experiments, this period of time might be too short for a drug to be metabolically activate in cells, hence it may be that RBS is also a direct-acting drug.

As mentioned previously anticancer drugs will give a greater fractional cell-kill when used against more rapidly growing tumors. In our experiments, only a subtle difference in cytotoxic effect of RBS on "normal" cells (SV-HUC-1) and tumor cells (T24) was

observed. This may be because either our experimental system is not sensitive enough to detect the difference or SV-HUC-1 cells themselves are not normal cells. Using a panel of tumor cells to examine the cytotoxic effect of RBS may give a more generalized picture of the effect.

Cytotoxic responses of human urotehlial cells after treatment with RBS were enhanced by laser light irradiation and were related to intensity (Fig. 4-1-6 & 7). Albumin bound RBS has less of a growth inhibitory effect (Fig. 4-1-8c). It was speculated that there were two possible mechanisms by which haemoglobin may rescue cells from cytotoxic effects of RBS: (1) RBS was bound to the haemoglobin therefore like albumin bound RBS the extent of the cytotoxic effect of RBS was decreased; (2) Light was absorbed by the haemoglobin thus the intensity of light delivered to RBS was decreased and therefore presented less of a potential cytotoxic threat. To rule out these two speculations, cells were treated with albumin bound RBS and the intensity of light was adjusted to be equal for each specimen. The results showed that haemoglobin has a protective capacity against the cytotoxic effect of RBS (Fig. 4-1-10).

The Turnbull reaction (Fig. 4-1-11) demonstrated that RBS is bound to cells. This phenomenon might explain why the albumin bound RBS had a reduced cytotoxic effect compared to unbound RBS. RBS binding directly to cells will release toxic molecules (eg. NO) directly into target cell whilst RBS bound to albumin will release toxic molecules into the environment which will subsequently perfuse into target cells. Thus higher concentrations of the drug are needed to achieve the same toxic effect.

RBS might release NO to carry out the blood vessel dilatory effects. This effect was enhanced by irradiation with laser light and was blocked by NO scavenger-haemoglobins (Flitney, F. W. *et al* 1992).
In our experiments the cytotoxic effects of RBS were enhanced by irradiation with laser light and were minimised by haemoglobin. Thus we might propose that NO may play a role in the mechanism of RBS cytotoxicity.

4-2 RBS causes DNA damage within target cells

4-2-1 Introduction

Cells which differ in radiosensitivity may be used to study the existence in cells of repair mechanism for damage caused by some anticancer drugs, and the relationship between DNA damage and loss of proliferating capacity. Chinese hamster ovary cell line (CHO-K1) and its X-ray sensitive mutants xrs, provide a particularly suitable cellular model (Dahm-Daphi, J. *et al* 1993). xrs-5 is one of six X-ray sensitive mutants which were derived from CHO-K1 cells by P. A. Jeggo (1983). The mechanism of x-ray sensitivity may differ from ataxia telangiectasia (AT) cells because xrs DNA synthesis after gamma-irradiation shows a greater degree of inhibition compared to the CHO cells (Jeggo, P. A. 1985). xrs-5 shows a moderate level of hypersensitivity to UV (254nm) light and methyl methanesulphonate (MMS) treatment. MMS will cause a large number of single strand breaks of DNA in the treated cells. The repair of these single strand breaks has been shown to be normal (Kemp, L. M. *et al* 1984). however there is a decrease in double strand break rejoining (Kemp, L. M. *et al* 1984; Dahm-Daphi, J. *et al* 1993) which may relate to an alteration of the organization of the DNA or the chromosome (Schwartz, J. L. *et al* 1990, 1993).

Cytochalasin B, a metabolite from the mold "Helminthosporium dematiodium" inhibits cytoplasmic cleavage and a number of processes concerned with cell movement. It is believed to interact with microfilaments (Wessells, N. K. *et al* 1971) or directly with plasma membrane (Estensen, R. D. *et al* 1971). In the presence of cytochalasin B nuclear division proceeds normally but cells fail to

separate completely and many consecutive nuclear divisions may take place without cleavage (Carter, S. B. 1967) however, DNA synthesis is inhibited very rapidly so that the vast majority of cells become binuclear and do not undergo further nuclear division (Kelly, F. *et al* 1973) but this DNA synthesis inhibition is not observed in SV 40 transformed cells hence they become highly multinucleated (Wright, W. E. *et al* 1972).

Micronuclei (MN) consist of acentric chromosome fragments or whole chromosomes that have not been incorporated in the main nuclei at cell division. Analysis of the frequency of micronuclei present in a given cell population has been recognised as a composite index of chromosome damage (Sternes, K. L. *et al* 1989). Induction of micronuclei can be a very simple and sensitive indication of repair deficiency when cells are exposed *in vitro* to DNA damaging agents (Arlett, C. F. *et al* 1989). M. Fenech (1985) use cytochalasin B to block the cytokinesis of dividing cells. Cells which have divided will accumulate as binucleates and scoring the micronuclei in these cells can overcome problems of mitotic delay or differences in cell cycle time and enhance the precision of the MN assay. CH0 and xrs-5 cells, were thus used to investigate the possible mechanism of action of RBS.

4-2-2 Procedures

4-2-2-1 Colony formation assay of CHO and xrs cells to quantitatively assess cytotoxicity of RBS

$2x10^5$/flask of CHO or $4x10^5$ /flask of xrs cells were grown in 25 cm^2 flasks, kept in a standard culture condition for 48 hours, allowed to attach and grown in exponential phase. Medium was removed and flasks were rinsed twice with PBS. Different concentrations of drug (RBS) dissolved in PBS were added (as a control PBS only was added) and flasks kept at 37°C for 1 hour. Flasks were rinsed twice with PBS and cells detached with TE. Cell numbers were obtained using a hemocytometer. An appropriate number of cells (200-10000) were plated in 90mm tissue culture grade Petri dishes with 10 ml of 10% FCS (v/v) Eagle's minimal essential medium and kept in a 5% CO_2 in air, 37°C fully humidified incubator. After 14 days the medium was poured out and the dishes were rinsed with PBS. Cells were fixed with methanol for 10 minutes and stain with 10% (v/v) Giemsa [BDH] in water.for 10 minutes and air-dried. Colonies containing more than 50 cells were scored as they were considered to originate from colony-forming cells Between 20 and 450 colonies were counted for each treatment. The percentage of survival was calculated by reference to controls. For each survival curve, triplicate dishes were seeded *per* treatment.

4-2-2-2 Micronuclei assay of RBS -treated CHO and xrs cells

$4x10^4$ CHO or $6x10^4$ xrs cells were grown in a 24 well flat bottom tissue culture plate and kept in a 5% CO_2 in air, 37°C, humidified incubator for 48 hours, allowed to attach and grow exponentially.

The plate was rinsed twice with PBS before 1 ml of 10 μM RBS in PBS was added to each well (control cells received PBS only). The plate was kept at room temperature for 40 minutes. RBS was removed and the plate rinsed twice with PBS. 1 ml of 10% FCS Eagle's minimal essential medium was added to each well then cytochalasin B (Cyto B; stock solution 3 mg/ml in DMSO) was added to give a final concentration of 3 μg/ml. The plate was kept in a 5% CO_2 in air, 37°C, humidified incubator for 16-24 hours. Cells were detached with TE and dispersed onto clean slides by centrifugation using a Shandon Cytospin 2 (800 rpm for 10 min). Slides were fixed in methanol for 10 minutes, stained in 56% (v/v) Jenner in water for 5 minutes and 25% (v/v) Giemsa in water for 10 minutes, with rinsing in water after each stain. Binuclear cells containing micronuclei clearly separated from and smaller than one-tenth of the main nucleus, were scored (Arlett, C. F. *et al* 1989). A total of 100 binucleate cells were scored *per* sample and at least 3 samples were determined *per* experiment. Results were expressed as the total number of micronuclei *per* 100 binucleate cells.

4-2-3 Result

4-2-3-1 Clonal assay of CHO and xrs cells to quantitatively assess cytotoxicity of RBS

Cells were treated with different concentrations of RBS for 1 hour, an appropriate number of cells were seeded in 90mm tissue culture grade petri dishes. Dishes containing 10 ml of serum supplemented medium were incubated for 14 days. Plates were stained and colonies were counted. Results showed that the survival of CHO and xrs cells after treatment with RBS was dependent on RBS concentration. A significantly increased cytotoxic effect of RBS was noted in xrs cells(Fig. 4-2-1). For example, a more than one log difference of survival between CHO and xrs cells when treated with 4μM of RBS.

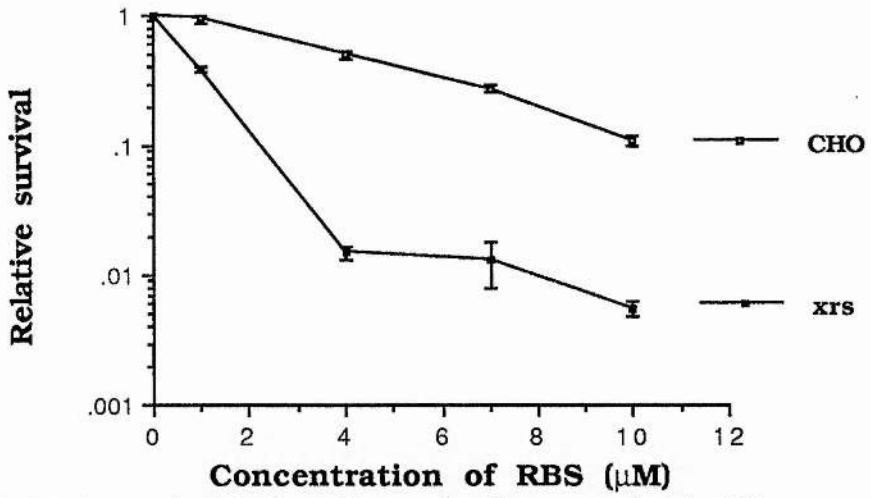


Fig. 4-2-1 Survival of CHO and xrs cells after treated with different concentrations of RBS for 1 hour.

4-2-3-2 Micronuclei assay of RBS-treated CHO and xrs cells

Further assessing the possibilities of DNA damage caused by RBS was carried out using the micronuclei assay. Cells grown in flasks

were treated with 10μM RBS (without drug treatment as controls) for 40 minutes in the dark. Drugs were replaced with fresh medium containing cytochalasin B and incubated for 16-24 hours. Cells were detached and dispersed onto clean slides. Micronuclei smaller than one tenth of the main nuclei were scored per 100 binuclear cells after cells were fixed and stained. Results in Fig. 4-2-1 showed xrs cells have higher back ground level of micronuclei formation. Furthermore a significantly increased number of micronuclei appeared in RBS-treated xrs cells. For example in one experiment, average micronuclei in 100 binuclear CHO cells was 4.57±0.33 which increased to 4.98±0.51 after 10 μM RBS treatment($p \leq 0.375$), while in xrs cells number of micronuclei changed from 9±0.71 to 17.64±0.79 ($p \leq 0.005$).

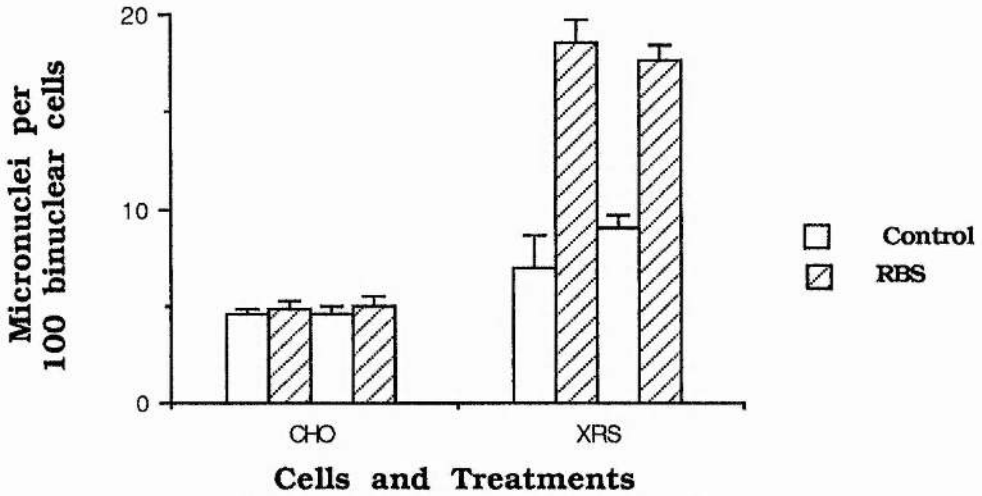


Fig. 4-2-2 Number of micronuclei per 100 binuclear cells. CHO or xrs cells were pretreated with 10 μM RBS for 40 minutes, cytokinesis was blocked with 3μg/ml of Cyto B. Controls were the same but without RBS pretreatment. Two separate experiments were performed and 3 samples were determined in each experiment.

4-2-4 Discussion

There are several mechanisms of action of anticancer drugs. Most drugs in current use inhibit cellular proliferation, commonly by inhibiting the synthesis of or directly damaging the DNA. Cells from most individuals are proficient in the repair of DNA damage exhibiting a variety of DNA repair systems. Studies of the effects of drugs on normal and a mutant variety of the same cell type deficient in DNA repair may provide evidence for the existence within cells of repair mechanisms. On the other hand they may provide an insight into the mode of action of the drug. One of the several methods by which NO is cytotoxic is through its ability to cause DNA damage [1-4-2-4]. In previous sections, indirect evidence has shown that NO might be responsible for the cytotoxic effect of RBS. By using CHO cells and its DNA repair deficient mutant xrs, it may be possible to demonstrate that RBS has a genotoxic effect on the DNA. For example if RBS only had an effect on cell membrane then survival of both cell types after treatment with RBS would show no significant difference, however if RBS had a DNA damaging effect then we might expect reduced survival in xrs cells.

The sensitivity to X-irradiation of CHO and xrs-5 cells was examined regularly to ensure the genetic stability relative to the appropriate phenotype of the cells was retained. The concentration of cytochalasin B required to yield optimal numbers of binuclear CHO or xrs cells was determined (both from personal communication with Dr. P. Johnston). Appropriate cells and [Cyto B] were used in these experiments.

The spontaneous or background level of micronuclei (MN) in binucleate cells gives an indication of genetic stability for each cell type. xrs had a higher back ground level of MN and furthermore a spontaneous loss of X ray sensitivity was noted in xrs-5 cells (Jeggo, P. A. *et al* 1983).

The origins of MN are varied from whole chromosomes to fragments of chromatids. Identifying centromeres in a MN may help to determine its origin. Knowing the origins of MN induced by drugs might predict the mode of action of MN-inducing agents. For example, colcemid binds to tubulin preventing microtubule assembly this inducing a high percentage of MN containing kinetochore (Thomson, E. J. *et al* 1988). It is not known if RBS induced MN are the result of acentric fragment formation or whole chromosome loss. If RBS is a spindle poison then the increasing frequency of MN should not be significantly different between these two cells types. However the result showed that xrs-5 cells had a significantly increased number of MN after treatment with RBS Fig. 4-2-2). Therefore we may speculate that RBS induced MN are caused by mechanisms other than spindle interference. Similarly, cell survival after the treatment of RBS was significantly lower in xrs cells. The possible mode of action of RBS associated cytotoxicity for cell killing may be due in part to DNA damage within target cells.

Conclusion

RBS has a cytotoxic effect on different human urothelial cells phenotypes. The level of cytotoxicity is related to drug concentration and the period of exposure to the drug . The growth inhibitory effect of RBS is enhanced by light irradiation and decreased by haemoglobin. This may indirectly indicate that nitric oxide is partly responsible for the cytotoxic effect of RBS.

Using colony growth in plastic plates and the induction of micronuclei as assays of cell damage caused by RBS. It was concluded that xrs-5 cells, a DNA repair deficient mutant of CHO cells, are more sensitive to RBS than its counterpart. This result held true for both assay techniques. This suggests that RBS may cause some kind of DNA damage within the target cells.

Future work including quantification of the level of NO causing a given level of cytotoxicity may provide more conclusive evidence as to the role of nitric oxide in the cytotoxic effect of RBS. A more detailed study into the nature of the differential response seen for CHO and xrs cells may provide an insight into the DNA damage which is caused by RBS.

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Appendix

Appendix I: Procedure of DNA fingerprinting.

Appendix II: Preparation of RBS.

Appendix III: Absorption spectrum of RBS.

Appendix IV: Published paper.

DNA Finger Printing

Genomic DNA Extraction

Materials

Lysis buffer
10% sodium dodecyl sulfate (SDS) [Sigma]
Proteinase K [Northumbria Biloogicals Limit]
25:24:1 phenol/chloroform/isoamyl alcohol(phenol/chloroform)
7.5M ammonium acetate [Fison U.K.]
TE buffer,pH 8.

Cell preparation

Confluent cells in flask harvest with trypsin or EDTA.
Suspend and spin cells twice with COLD PBS (spin at 1000 rpm for10minutes).
Do cell count at second wash for approximate cell number.
Remove PBS and stand the cell pellet on ice.

Cell lysis and protein digestion

Resuspend cells in lysis buffer evenly.(1ml lysis buffer per1.2-2.0x10^7 cells)
Aliquot into 1 ml in eppendorfs.
Add 100µl proteinase K at 10mg/ml.
Add 250µl of 10% SDS. Mix with cut off blue tip.
Incubate overnight with gentle shaking at 50° C tube cover with paraffin film to prevent incidental leaking.

Extraction of nucleic acids

Do the measurement of volume of lysant by turning the scale of pipetman.
Extract with an equal volume buffered phenol, mix gently, centrifuge at 13000rpm for 5 minutes.
Prepare a tube with half original volume of phenol and half original volume of 24:1 (v/v) chloroform : isoamy alcohol.(phenol/chloroform)
Remove as much as possible of top layer clear fluid into the above prepared tube, mix and centrifuge at 13000 rpm for 5 minutes.
Remove top layer and repeat extraction with half volume phenol/chloroform-isoamy alcohol once again.
Remove top layer and extract with one volume chloroform.

Purification of DNA

Transfer the top aqueous layer to a new tube and add half original volume of 7.5M ammonium acetate and 2 volumes 100% icy cold ethanol.
DNA should precipitate immediately.
Hook out the DNA ppt. into a eppendorf containing 70% ethanol.
Rinse twice with 70% ethanol and squeezing on side of tube.
Lift into clean eppendorf with minimal ethanol and leave to dry about an hour in fume hood.
Dissolve it in 100µl of TE pH7.6 overnight at 50°C water bath.

Reagents and solutions

Lysis buffer:100mM NaCl, 10mM Tris.Cl, pH 8 [Sigma], 25mM EDTA, pH 8
TE solution: 10mM Tris.Cl , 1mM EDTA, pH 8
7.5M ammonium acetate : dissolve 289.05g of ammonium acetate in 150ml H_2O, add H_2O to 500ml
Buffering phenol
Add 0.5g of 8-hydroxyquinoline[Sigma] to a 2-liter glass beaker containing a stir bar.
Gently pour in 500ml of liquified phenol or melted crystals of redistilled phenol.[Aldrich, UK]
Add 500ml of 50mM Tris base.
Stir 10 minutes at low speed with magnetic stirrer at room temperature.
Let phases separate at room temperature.
Gently decant the top(aqueous) phase into a suitable waste receptacle.
Add 500ml of 50mM Tris-Cl, pH8.
Repeat stir and decant aqueous phase.
Add 250ml of 50mM Tris-Cl, pH8, or TE buffer and store at 4°C in light-tight bottle.
For use in DNA purification procedure, mix 25 volume phenol with 24 volume chloroform and 1 volume isoamyl alcohol[Sigma].

Agarose minigel electrophoresis

Materials
Electrophoresis buffer (TBE)
Ethidium bromide solution
Electrophorisis-grade agarose
DNA molecular weight markers.
30 wells Biorad horizontal minigel apparatus.

Preparing the gel
0.8gms of agarose(Agarose 15 Eletron) dissolve with 80 ml of TBEx1 in microwave oven , add ethidium bromide1µg/ml of agarose.
Seal the open ends of gel casting platform with cellatape.
Level the gel tray.
Pour in the melted agarose while it cool to 55°C and insert the gel comb.
After the gel has hardened, remove the tape from the open ends of the gel platform and withdraw the gel comb carefully.
Place the gel casting platform containing the set gel in the electrophoresis tank.
Add sufficient electrophoresis buffer(TBEx1) to cover the gel to a depth of about 1 mm , make sure no air pockets are trapped within the well.

Loading and running the gel
3 µl glycerol dye and 2 µl DNA sample load into each well. Avoid mixing of samples between wells.
Load a well with known concentration of DNA as a reference.

Check the leads are attached correctly so that the DNA will migrate into the gel toward the anode or positive lead.
Run the gel at 80-100 volt for 45 minutes.Turn off the power.
View the gel above the ultra-violet light, this show if the DNA is degraded as well as its concentration.

Reagents and solutions

Glycerol dyes: 50% glycerol[Sigma], 100mM EDTA pH 8.0[BDH,disodium salt], 0.1% xylene cyanol[Sigma], 0.1% bromophenol blue[BDH], water to volume.
10x TBE for 1 liter: 109g Tris [BDH], 9.3g EDTA , 55g Boric acid [BDH, HBO_3]
10mg/ml ethidium bromide: Dissolve 0.2g ethidium bromide[Sigma] in 20 ml H_2O mix well and store at 4°C in dark.

Measure the concentration of DNA by Fluorometer

Materials

Machine: Hoefer Scientific Instrument (San Fransisco USA), DNA Fluorometer Model :TKD 100.
Working dye solution
DNA molecular weight marker

Calibration

Wear gloves to handle the cuvette well.
2 µl of standard DNA and 2 ml of working dye solution add into the cuvette Calibrate the machine accordingly.
Pour out the standard solution and rinse cuvette with 1 ml working dye solution.

measure

2 ml working dye solution in cuvette should read zero if measurement is too high then rinse cuvette again until it read zero.
Mix 2 µl of sample DNA and 2 ml of working dye solution and read the concentration.

Digestion of DNA with restriction endonucleases

Materials

Restriction endonucleases (HeaIII), and (10x) restriction endonuclease buffer[Gibco]

Methods

After run the minigel, knowing the quality and quantity of sample DNA then start to cut the DNA with restriction endonuclease.
If the concentration is in the usual range(250-500µg/ml).
Use 60 µl of sample DNA add

8 µl of 10x salts
8 µl of BSA (1mg/ml)
4 µl of Hae III

Mix by taking up and down in pipette and leave in 37°C incubator overnight.

Reagents and solutions

10x Hae III buffer: 0.1ml of 5M NaCl, 0.06ml of 1M TrisHCl (pH7.4), 0.06ml of 1M $MgCl_2$, 0.01ml of 1M dithiothreitol (DTT), 0.67ml of distilled water

Extract digested DNA

Materials

Same as genomic DNA extraction except lysis buffer.

Extract DNA

Add sample volume of phenol/chloroform to digest mix and spin at 13000 rpm for 5 minutes.

Remove supernatant to eppendorf containing sample volume of chloroform, mix and spin at 13000 rpm for 5 minutes.

Purification of DNA

Remove supernatant to eppendorf containing 1/10 volume of 2.75M sodium acetate and 3x volume of cold 100% ethanol.

Spin to pellet DNA, pour off ethanol and wash with 70% ethanol.

Remove as much as ethanol as possible and dry in vacuum.

Add 20µl of TE to sample and leave to dissolve.

Test for digestion and concentration of DNA

Test DNA for complete digestion in minigel.

Measure concentration.Ether on the minigel or use DNA fluorimeter.

Dilute, if necessary, the concentration to some standard.(500 µg/ml). This allows the loading the same volume into every well.

Agarose Gel Electrophoresis

Materials

Same as minigel electrophoresis

Instrument: 20 wells 20cm x 20cm gel size horizontal electrophoresis apparatus

Preparing the gel

2.4gms of agarose(Agarose 15 Eletron) in a beaker add 300ml of TBEx1 mark the level of fluid.

Cover with paraffin film with few hole on it.

Melt it in microwave oven make sure agarose powder well dissolved.

Add MQ water back to original water level.

Seal the open ends of gel casting platform with cellatape.

Level the gel tray.

Pour in the melted agarose while it cool to 55°C and insert the gel comb.

After the gel has hardened, remove the tape from the open ends of the gel platform and withdraw the gel comb carefully.

Place the gel casting platform containing the set gel in the electrophoresis tank.

Add sufficient electrophoresis buffer(TBEx1) to cover the gel to a depth of about 5-10 mm , make sure no air pockets are trapped within the well.

Loading DNA and running gel

Load wells with equal amount of sample DNA and dye.There should be 1/3 volume dye in each well.Avoid mixing samples between wells.

Lambda ladder marker and undigest probe DNA run along side with sample DNA.

Leave samples to equilibrate with buffer for 10 minutes before running gel.

Check the leads are attached correctly so that the DNA will migrate into the gel toward the anode or positive lead.

Run the gel at 24 volts for 48 hours.

Stop the power supply and change the whole running buffer solution.

Add 50 µl of 10mg/ml ethidium bromide into 1 liter of running buffer solution.

Run the gel at 36 volts for 24 hours.

Check the gel on a transilluminator and record the migration distance of each ladder.

Reagents and solutions

Lambda ladder marker[Gibco]

Lambda phage DNA digested with EcoR I gives a useful set of bands with a band at 40,90,112,116,132,180 Kbp.

For 2 wells : 10µl lambda DNA (concentration is about 500µg/ml)

1µl of EcoR I[Gibco]
2µl of 10x salts
7µl of distilled water

Mix, spin down and put at 37°C for 1.5-2 hours.

Add 20µl of glycerol dyes and split between two wells.

Southern Blotting

Materials

UV transilluminator
0.25N HCl
Gel Soak 1 solution(denaturation solution)
Gel Soak 2 solution(neutralization solution)
SSC solution
Glass or metal decanting trays
Nitrocellulose membrane(Zeta-Probe GT, BIO-RAD
Whatman 3MMfilter paper

Methods

Check condition of lanes of DNA on UV transilluminator.

Measure the position of the lambda bands from the well.

Soak gel in 0.25N HCl and rock the tray gently for 20 minutes depurination of DNA.

Pour off the HCl rinse the gel with distilled water, then immerse in Gel Soak 1(1x) and rock the tray gently for 45 minutes. Denaturation of DNA.

Pour off the Gel Soak 1 rinse the gel in distilled water and immerse gel in Gel Soak 2 (1x) and rock the tray gently for 45 minutes .Neutralising of the gel.

Put a piece of glass larger than the gel in a plastic tray raised off the tray.

Lay a wick of Whatman 3MM paper over the glass(wider than glass) so both ends reach into the SSC. pour about 1L of SSC(10x) over the wick and roll out any air bubbles with a 10ml pipette. The 3 MM paper should now form a wick.

Lift the gel out of the Gel Soak 2 and turn over and place on the wick. Remove air bubbles trapped between gel and wick by rolling a pipet over gel as above. It is essential that the gel be turned over. The DNA is near the bottom.

Cut a piece of membrane (Zeta-Probe GT Boltting membranes,BIO-RAD) slightly smaller than the gel. Do not touch it without wearing gloves.

Soak membrane in distilled water and place on top of gel. Remove trapped air bubbles as above.

Cover the gel with cling film leaving only the membrane uncovered.

Soak 2 pieces of 3MM paper, cut slightly small than the membrane, in SSC(10x) and put on top of membrane.Make sure that the piece of 3MM paper does not overhang the membrane and remove the trapped air bubble as above.

Cover up the ends of tray with plastic wrap to minimize evaporation during the transfer.

Add paper towels being careful to avoid any chance of a short circuit.

Paper towels are placed deep enough that they keep up a steady capillary pressure and do not become saturated.

Change the paper towels every 5 minutes for the first 15 minutes, thereafter every 15 minutes for the first hour, and then leave overnight for 19 hours or so.

After the transfer is completed, take apart the pyramid so that the membrane is still lying on the gel. On the membrane mark the position of the wells and date and number the membrane using a very soft pencil.

Using blunt forceps, remove the membrane and place it in a tray of SSC(2x) for 5 minutes to wash away debris.

Air dry for 20 minutes on a piece of filter paper, then bake in a pre-heated vacuum oven for 30 minutes at 80°C in a piece of folded 3MM paper.

Reagents and solutions

Gel Soak 1 (GS1) [5x for 2 liters]: 355.5g NaCl, 80g NaOH.

Gel Soak 2 (GS2)[2x for 2 liters]:142.2g NaCl, 484.4g Tris pH 8, pH with HCl takes about 200 ml.

SSC (20x for 1 liter): 175g NaCl (3M), 88g $Na_3citrate.2H_2O$ (0.3M), pH to 7.0 with 1M HCl.

0.25 N HCl: 21.5ml of concentrated HCl in 2 liters of distilled water.

Making the probe

The plasmid probe DNA is gifted by Dr. David Parkin's Lab Nottingham.

Briefly describe the characteristic of the DNA

A Pair of transcribable vectors pSPT 18 and pSPT 19 (from BCL) were selected which contained opposed RNA polymerase promoters from T7 and SP6 phages flanking the pUC18 multiple cloning site. They also conferred ampicilin resistance, and are multicopy.

Minisatellite regions of 33.6 and 33.15 were liberated by sequential digestion of M13 RF DNA with Hind III and EcoR I, purified by electroelution and force cloned into the Hind III/EcoR I site of pSPT 18 and pSPT19 to yield four recombinants (pSPT 18.6, 19.6, 18.15, and 19.15) permitting either strand of either probe to be prepared, by selecting T7 or SP6 polymerase for transcription. The recombinants were transformed by the CaCl2 method into E. Coli. DH1 a recombination deficient host strain to reduce the possibility of rearrangements. Recombinants were selected by ampicillin.

Isolation of plasmid from E. Coli. (Stephen D. et.al.1990.)

Materials

GTE solution
LB medium
0.2M NaOH
1% SDS
3M Potassium acetate pH 4.8
absolute ethanol
70% ethanol
TE buffer

Methods

Scrape the frozen surface of glycerol stock with a sterile inoculation wire, and streak onto a fresh LB ampicillin plate. Incubate the plate overnight inverted at 37°C.

Inoculate 5 ml LB ampicillin broth with a single colony from a fresh plate. Incubate at 37°C with gentle agitation overnight. Prepare in duplicate.

Decant 1.6ml of the overnight culture into an Eppendorf tube, and spin in a benchtop centrifuge at 13,000 rpm for 2 minutes.

Completely remove the supernatant from the bacterial pellet, by tipping and draining.

carefully resuspend the pellet in 180 µl GTE solution.

Add 360 µl of a freshly prepared solution of 0.2M NaOH/1% SDS, invert ten times before placing on ice for 5 minutes.

Add 270µl of 3 M potassium acetate (pH 4.8), invert ten times before replacing on ice for 5 minutes. Do not vortex.

Centrifuge at 13,000 rpm for 5 minutes, and remove supernatant to a clean 1.6ml Eppendorf tube.

Add 1 volume (800µl) of absolute ethanol to the supernatant, vortex briefly, and immediately centrifuge at 13,000 rpm for 5 minutes.
Carefully discard the supernatant, and wash the plasmid pellet with 1 ml of 70% ethanol,before a final certrifugation at 13,000 rpm for 2 minutes.
Discard the supernatant, and dry the pellet under reduced pressure.
Resuspend the DNA pellet in 20 µl of sterile TE.

Reagent and solutions

GTE solution: 25mM Tris-HCl, pH 8.0, 10mM EDTA, 50mM glucose.
LB medium /liter, agar plate , + ampicillin: 10g Bactotryptone [Difco], 5 g Bacto yeast extract [Difco], NaCl 10g. Adjust pH to 7.0 with 5N NaOH, for plates, and add 15g bacto-agar before autoclaving. Add ampicillin to a final concentration of 35-50 µg/ml.

Preparation of RNA probe (follows Carter, R. *et al* 1989)

Linearize the vector

3µl (1µg) of uncut plasmid DNA
1 µl of EcoR I for 19.6 or Hind III for 18.15 probe
0.6µl 10x salt solution
1.4µl of sterile distilled water
Spine down and incubate in 37°C water bath for 1.5-2.5 hours.

Transcription reaction

Add to the linearized abstracted DNA with
4 µl of 5x transcription buffer
3µl of NTPs (10mM each of ATP,GTP,UTP, 1mM CTP)
2µl of DTT (100mM)
1µl of RNAsin (25u/µl)
3µl of 32P CTP (400 Ci/m mol)[Ameshan]
1µl of T7 RNA polymerase (20U/µl)
Spin down and incubate in 37°C for 1 hour.

Removal of incorporated nucleotide

Stop the reaction with 20µl of nick stop mix.
Through a 1-ml column of Sephadex G-50 [Pharmacia, Sweden] equilibrated with TE to remove unincorperated nucleotides.
Count the radiation activity of the probe with liquid scintillation analysis [Tricrab 1600TR canberra packard USA].

Reagents and solutions

Transcription buffer (x5) 200mM Tris-HCl pH 7.5, 30mM MgCl2, 10mM spermidine, 50mM NaCl.
RNAsin : (Amershan, N120Y).
T7 RNA polymerase :[pharmacia].
NTPs, DTT, transcription buffer: (from Promega Riboprobe Gemini II kit).
Nick stop mix: 0.9%(w/v) blue dextran [Sigma], 0.03%(w/v) bromocresol purple [BDH] 20mMEDTA.

Spin-Column procedure for separation radioactively labeled DNA from unincorporated NTP precursors

Materials

TE buffer

6.5 in. Pasteur pipet

Glass wools welled column resin (Sephadex G-50 or Biogel P-60 ,BIO-RAD)

1 ml syringe

Plug the bottom of a 1-ml disposable syringe with clean glass wool.

Swirl the column resin to make an even suspension, and fill the syringe with the suspension.

place the syringe containing the resin into a polypropylene tube that is suitable for centrifugation in a desktop centrifuge.

Spin at 3000 rpm for 3 minutes in order to pack the column.

Load the sample in the center of the column and spin at 3000 rpm for 3 minutes.

Rinse the sample tube with 2 volume of TE and reload into the column.

Spin at 3000 rpm for 3 minutes. Collect all the liquid at the bottom of the tube containing the labeled RNA.

Count the radiation activity of the probe.

Prehybridizing, hybridizing, and washing the filter

Materials

BLOTTO solution

SDS

SSC solution

hybridization chamber

Methods

Make up prehybridization/hybridization fluid(e.g.1% BLOTT ,2% SDS ,1x SSC)

Prehybridize the membrane at 64°C over night.

Add up to $6x10^7$ cpm RNA probe to the hybridization chamber. hybridize at 64°C over night.

wash the membrane at 65°C on shack water bath for 45 minutes with several changes of wash solution(e.g. 1x SSC,0.1%(w/v) SDS)

The damp membranes are wrapped in saranwrap

Exposed to X-ray film for 2 days with two intensifing screens followed by 14 days without screen.

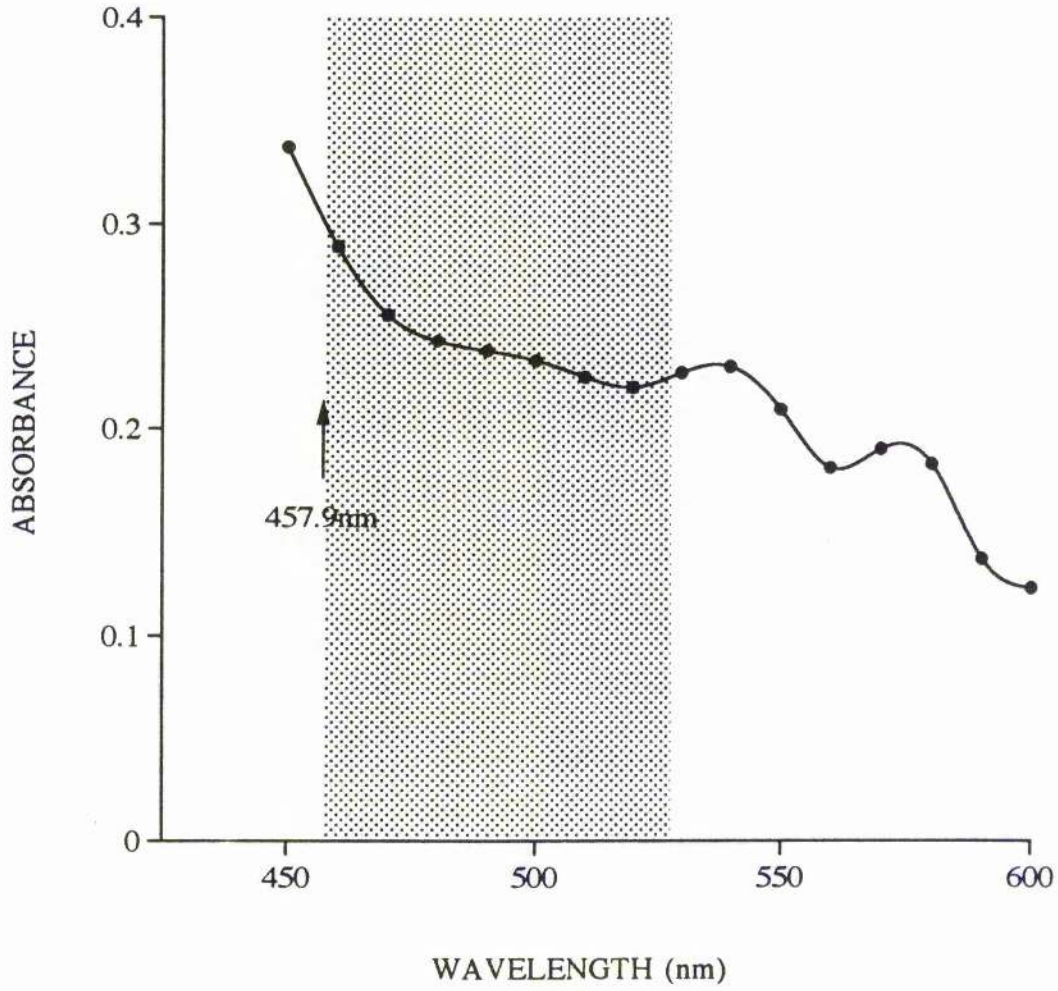
Reagents and solutions

BLOTTO solution (100ml of 10% solution): 10g of non-fat posered milk (Marvel), 0.2g of sodium azide, Add 10 µl diethylpyrocargonate (DEPC), Incubate at 42°C for 4-6 hours to remove DEPC.

Preparation of Roussin's black salt

Roussin's black salt (RBS), Sodium heptanitrosyl-tri-μ3-sulphido-tetraferrate: $Na[Fe_4S_3(NO)_7]$

Sodium nitrite ($NaNO_2$) and sodium sulphide (Na_2S) were stirred and heated under nitrogen until boiling. Ferrous sulphate solution ($FeSO_4$) was added from a dropping funnel (DE) to the accompaniment of black frothing, quelled by the addition of 20% ammonia solution. The resulting mixture was hot-filtered through pre-heated Hyflo to remove iron oxide and the residue washed with hot/boiling water. The filtrate was cooled on ice and crude RBS precipitated out of solution. The precipitate was filtered off, recrystallized from hot water and dried in a vacuum desiccator. RBS was stored in the dark, under nitrogen and at -10°C. Purity was assessed using infra-red spectroscopy where three peaks are seen at 1795,1747 and 1707 cm^{-1}.



Absorption spectrum of RBS (10^{-4}M). Shaded area shows range of wavelenghts available using argon-ion laser (Spectra Physics model 168-09). The figure shows 457.9nm to be the optimal available wavelength for irradiation of RBS.

7.6 **THE LEVEL OF DETECTABLE STEM CELL FACTOR RELEASED BY A HUMAN UROTHELIAL CELL LINE IS REDUCED WITH THE DEVELOPMENT OF TUMORIGENICITY.**

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We report the development of a tumorigenic human urothelial cell line from a non-tumorigenic cell line and the observation of a reduction in the level of detectable Stem Cell Factor (SCF) released by these cells.The SV-HUC-1 cell line was derived from human urothelium immortalized by infection with wild-type Simian Virus 40 (Christian, B.J. et al, 1987, Cancer Research 47 : 6066). This cell line did not produce tumours when innoculated into athymic mice, but if these cells were grown for 6 months in chemically-defined culture medium without serum, hormones, or growth factors, a new variant of the cell line was selected which did produce tumours. The tumours appeared after 20 weeks and were present in 4/20 mice. Tissue explant culture of these tumours created a continuously passaged cell line. Subsequent injection of these cells into athymic nude mice produced tumours in all the recipients. The levels of human stem cell factor (SCF) in medium conditioned by tumorigenic and non-tumorigenic cells were quantified by means of enzyme linked immunosorbant assay (Quantikine kit). The results indicate that the cells released less SCF when they became tumorigenic.

The biological activity of SCF can be assayed by cloning haematopoietic cells in soft agar in combination with other cytokines when high proliferative potential colony forming cells (HPP-CFC) are stimulated to produce large colonies. Conditioned medium prepared from the cell lines acts synergistically with rM-CSF (macrophage colony stimulating factor) and rGM-CSF (granulocyte-macrophage colony stimulating factor) to produce large colonies from cloned bone marrow cells. These large colonies are not produced by any of the factors singly. Similar colonies are produced in the presence of rSCF plus rM-CSF plus rGM-CSF. It thus seems likely that human urothelial cells in culture can produce biologically active SCF.

It has been proposed that the development of tumorigenicity is a multi-step process and SCF, which is reported to prevent apoptosis (programmed cell death), may play a possible role in this process.

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